A comparative study of levels of methylglyoxal and reduced glutathione in different organs of rats treated with high carbohydrate diets

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By

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ABSTRACT

Methylglyoxal (MG) is a reactive dicarbonyl compound mainly formed during glucose and fructose metabolism. Diabetic patients have increased plasma levels of MG. Our laboratory has shown that treatment with MG induces insulin resistance and type II diabetes in male Sprague-Dawley rats. However, the increases in endogenous MG level attained in different organs and its contribution to the pathogenesis of diabetes following the administration of either high glucose or high fructose diet have not been addressed. The present study aims to investigate whether the harmful effects induced by increased consumption of glucose and/or fructose is linked to increased MG generation. *In vitro* studies have suggested that L-arginine is an effective MG scavenger. Accordingly, another goal is to determine whether L-arginine pretreatment would scavenge MG under *in vivo* setting and reduce the harmful effects of hyperglycemia. MG and reduced glutathione (GSH) levels were determined in plasma and urine and in different organs of male Sprague-Dawley rats after 12 weeks of treatment with either high fructose or high glucose diet. GSH plays an important role in the degradation of MG and bears an inverse relationship with the levels of MG. The key results obtained suggest that both diets significantly increased blood pressure and plasma MG levels. A high fructose but not a high glucose diet, increased the plasma total cholesterol, triglycerides levels and total cholesterol/HDL ratio in parallel with the increases in MG and GSH levels in the liver. Increased MG levels seen in both aorta and mesenteric artery induced by high glucose or fructose diet was attenuated by pretreatment with L-arginine. These findings suggest that elevated MG level induced by treatment with high carbohydrate diets in both conduit (aorta) and resistance type (mesneteric artery) vessels may be linked to endothelial dysfunction seen in hyerglycemic/diabetic states. High glucose but not high fructose diet significantly increased MG levels in the pancreas. This observation is consistent

with the well-known glucotoxicity caused by hyperglycemia in the pancreas. Taken together, these data provide the first evidence that elevated MG levels in certain organs/tissues following consumption of high fructose and/or glucose diet(s) may play a critical role in contributing to the metabolic abnormalities and the endothelial dysfunction that precedes the onset of macro and microvascular complications in either hyperglycemic and/or type II diabetic states. Interestingly, quenching of elevated MG levels in tissues by pretreamtent with L-arginine overcomes MG-induced vascular damage and endothelial dysfunction caused by high fructose and high glucose diet regimens.

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DEDICATION

To my beloved brother

Gary Sandhawalia

Because for the world he might have left, but for me, he is forever by my side as my biggest strength and support never ceasing to love, care and guide me…

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation end products
ALA	Alagebrium
AMO	Acetol/acetone monooxygenase
ATP	Adenosine triphosphate
BMI	Body Mass Index
BP	Blood pressure
CEA	Carboxyethylarginine
CEL	Carboxyethyllysine
CML	Carboxymethyllysine
DHAP	Dihydroxy acetone phosphate
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra acetic acid
EI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal stage
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GTT	Glucose tolerance test
HDL	High density lipoproteins
HFCS	High fructose corn syrup
HPLC	High-performance liquid chromatography
ICAM-1	Intercellular adhesion molecule 1

IFNγ	Interferon gamma
IL	Interleukins
JNK	c-Jun N-terminal kinase
L-Arg	L-Arginine
LC	Liquid chromatography
LDL	Low density lipoproteins
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase
MC	Mass chromatography
MG	Methylglyoxal
MODIC	Methylglyoxal-derived imidazolium cross-linking
MOLD	Methylglyoxal-lysine dimer
MTP	Microsomal triglyceride transfer protein
NAC	N-acetyl cysteine
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa beta
NO	Nitric oxide
NOS	Nitric oxide synthase
OCD	Ornithine decarboxylase
PCA	Perchloric acid
PTB	Phenacylthiazolium bromide
RAGE	Receptor for AGEs
ROS	Reactive oxygen species

SHR	Spontaneously hypertensive rats
SREBP-2	Sterol regulatory element binding protein-2
SSAO	Semicarbazide sensitive amine oxidase
T2DM	Type 2 diabetes mellitus
THP	Tetrahydropyrimidine
VCAM1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VLDL	Very high density lipoproteins
VSMCs	Vascular smooth muscle cells

INTRODUCTION

The problem of diabetes

Diabetes is a metabolic disorder in which a person becomes hyperglycemic due to a defect in insulin secretion or action or both of these (Lu et al, 2011). Type II Diabetes can vary from a predominant insulin resistance with relative insulin deficiency to a predominant defect in insulin secretion with relative insulin resistance. Chronic poorly controlled hyperglycemia of diabetes has many significant long term complications which are the main cause of morbidity and mortality from this disease (Canadian-Diabetes-Association, 2008). Maintaining optimum glucose control reduces the risk of long term complications of diabetes.

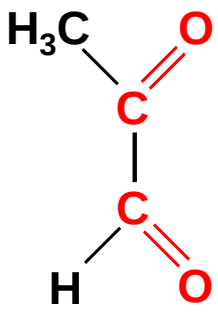
In 1985, an estimated 30 million people around the world were diagnosed with diabetes; in 2000, that figure rose to over 150 million; and, in 2012, the International Diabetes Federation (IDF) estimated that 371 million people had diabetes (International-Diabetes-Federation, 2012). This number is estimated to increase to 552 million (or 1 in 10 adults) by 2030, which means 3 new cases per second (International-Diabetes-Federation, 2012) . Although the data suggests that developing countries will be the ones most impacted, Canada will be hit as well. As of 2009, the estimated prevalence of diabetes in Canada was 6.8% of the population i.e. 2.4 million Canadians which is a looming 230% increase compared to prevalence estimates in 1998. By 2019, that number is expected to grow to 3.7 million (Diabetes-in-Canada, 2011). Diabetes is the leading cause of blindness, end stage renal disease (ESRD) and non-traumatic amputation in Canadian adults and as such, the complications are far worse than the disease itself.

Recent studies have pointed out that a chemical compound known as methylglyoxal (MG) might have a role in the causation of diabetes and/or diabetic complications. This is formed

endogenously mainly during the glucose metabolism in body but has other sources as well (Kalapos, 1999). Studies have shown that the levels of methylglyoxal are elevated in patients of T2DM and also co-relate with the levels of HbA_{1c} and fasting glucose (Nemet et al, 2005). It has been also suggested that the increased levels of MG cause the impairment of renal mitochondrial functions and lead to the causation of diabetic nephropathy (Rosca et al, 2002) through the mechanism of oxidative stress (Rosca et al, 2005).

METHYLGLYOXAL

MG is a α-dicarbonyl compound (Fig. 1) which is produced as a by-product of various chemical reactions (Matafome et al, 2013). Chemically, it is also called pyruvaldehyde, pyruvic aldehyde or 2-oxopropanal. It is a yellow liquid with a characteristic pungent odour. It has a low molecular weight of 72.06.



Methylglyoxal ($C_3H_4O_2$)

M.W. = 72.06

Figure 1. Chemical structure of methylglyoxal.

Synthesis

MG is produced endogenously during the breakdown of carbohydrates, lipids as well as amino acids and involves both enzymatic and non-enzymatic reactions (Kalapos, 1999). MG is generated from the glucose metabolism through the glycolytic pathway (Phillips & Thornalley, 1993). Here, MG is produced by the non-enzymatic fragmentation and elimination of phosphate from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (DHAP) (Fig. 2) (Thornalley, 1996).

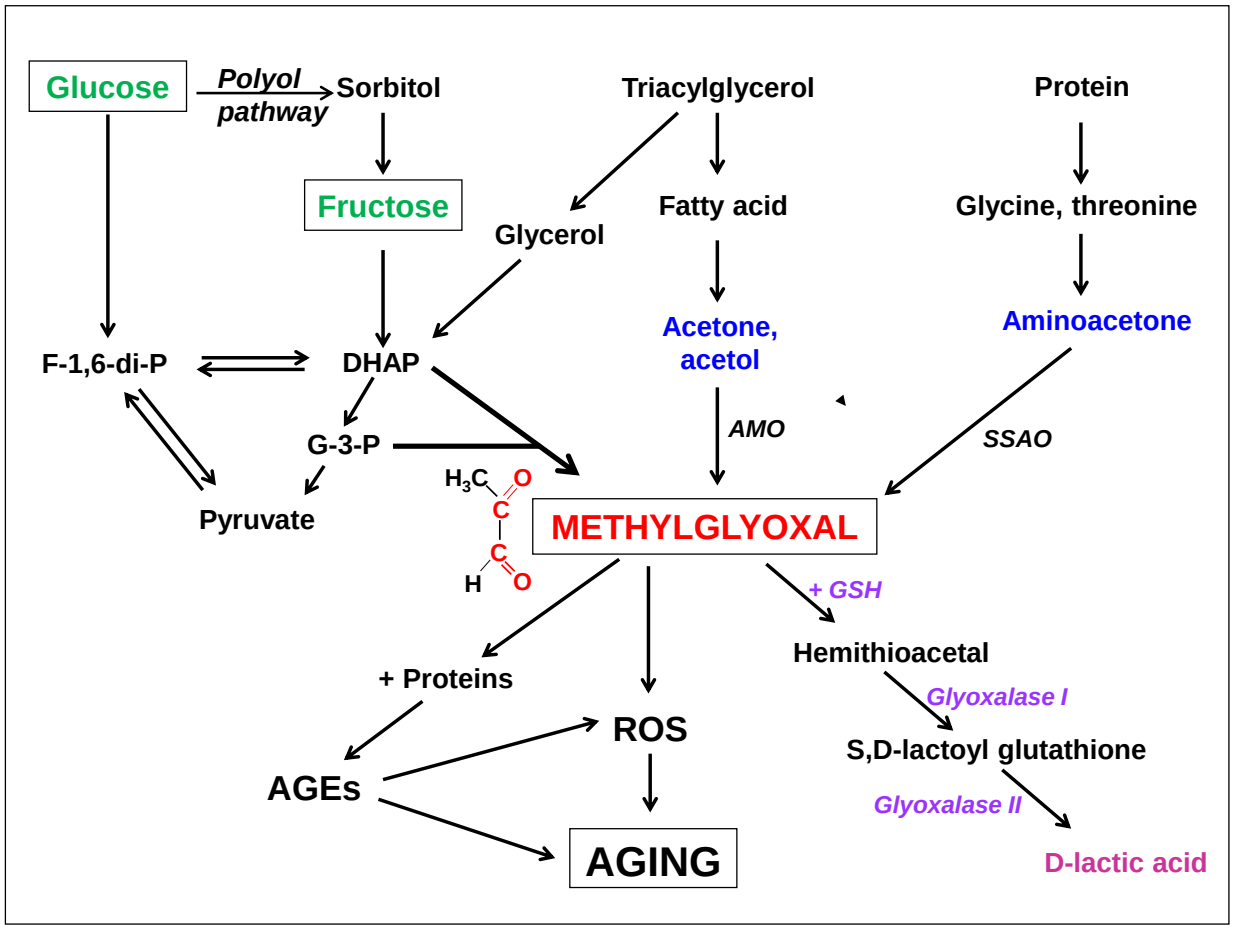


Figure 2. Synthesis of methylglyoxal. [Figure with permission from: (Desai et al, 2010)]. Methylglyoxal (MG) is mainly generated from triose phosphate intermediates of glucose and

fructose metabolism. Other minor sources are acetone/acetol and aminoacetone produced during fatty acid and amino acid metabolism, respectively. Abbreviations: AMO, acetol/acetone monooxygenase; DHAP, dihydroxy acetone phosphate; F-1,6-di-P, fructose-1,6-diphosphate; G3-P, glyceraldehyde-3-phosphate; ROS, reactive oxygen species; SSAO, semicarbazide sensitive amine oxidase.

Alternatively, MG is also produced by lipid metabolism which involves either enzymatic or non-enzymatic reactions in which glycerol, acetoacetate or acetone are converted to 1,2-dicarbonyl. MG formation also occurs during the lipid peroxidation process (Shibamoto, 2006). The metabolism of amino acids (mainly threonine and glycine, and partially tyrosine) is also connected with synthesis of MG (Thornalley, 1993). Aminoacetone is formed as a product of threonine & glycine metabolism and its conversion into MG is catalyzed by semicarbazide-sensitive amine oxidase (SSAO) (Yu et al, 2003).

Degradation of MG

MG is currently believed to be a toxic compound and the glyoxalase system is responsible for its detoxification in the body (Sousa Silva et al, 2013). The glyoxalase system consists of two enzymes, glyoxalase I & glyoxalase II, and catalytic amount of reduced glutathione. Glyoxalase I catalyses the formation of S,D-lactoyl-glutathione from hemithioacetal formed non-enzymatically from MG and reduced glutathione (Fig. 3).

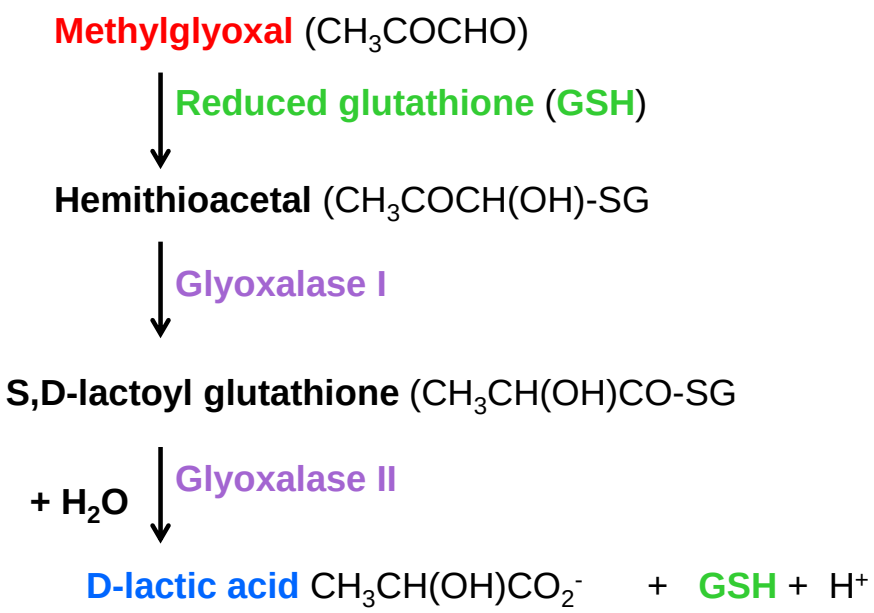


Figure 3. Degradation of methylglyoxal by the glyoxalase enzymes.

Glyoxalase II hydrolyses this S,D-lactoyl glutathione to D-lactic acid and hence, reforms the reduced glutathione consumed in the first step (Thornalley, 1996).

Levels of methylglyoxal

MG is a highly reactive compound. This is indicated from a reported estimate which states that about 99% of MG in the body is bound to proteins and other biomolecules whereas only about 1% is in free form (Chaplen et al, 1998). Hence, accurate measurement of levels of MG in plasma and other body compartments is not an easy task. Judging by this, the actual concentration of MG should be much higher than the ones reported by various studies since most of them only calculate the unbound free form of MG.

The normal plasma levels of MG have been reported to be in the range of 0.2-1 μM (Nemet et al, 2004). In another study, the normal plasma levels of MG have been determined to be

around 0.5-1.5 μM (Jia & Wu, 2007; Wang et al, 2007) which is only a slight variation. It has been shown that the levels of MG rise by about 2 to 4 times in the plasma of diabetic patients (Wang et al, 2007).

The concentration of MG in human blood samples has been found to be about 80 pmol/g. In terms of pathophysiology, such low concentration is protective because of the high reactivity of α-carbonyls with nucleic acids and body proteins (Thornalley, 1998). Not much variation was, however, found in MG concentration between male and female plasma samples.

In another study, the concentration of MG in human lens has been calculated to be in the range of 1.78±0.84 nmol (g wet weight)$^{-1}$. The concentration of MG was, thus, found to be about 20 times higher than the concentration in the blood samples from normal human subjects (Haik et al, 1994).

Another study reports that the normal concentration of MG in the urine of healthy subjects is around 15 μM (Espinosa-Mansilla et al, 1998). A male to female variation in the urinary concentration of MG was reported by another study. The MG levels were determined in the form of a methylglyoxal/creatinine ratio. For women, the value estimated was 0.60±0.22 μg/mg of MG/Creatinine whereas for men, it was 0.49±0.05 μg/mg. These results corresponded with the BMI of the individuals (Espinosa-Mansilla et al, 2007).

One interesting study compares the levels of MG in plasma samples of normal and diabetic subjects. The outcome observed was that the average amount of MG in serum of normal subjects was in range of 0.025-0.065 μg/ml whereas in diabetics, it was in the range of 0.115-0.228 μg/ml (Kandhro et al, 2008).

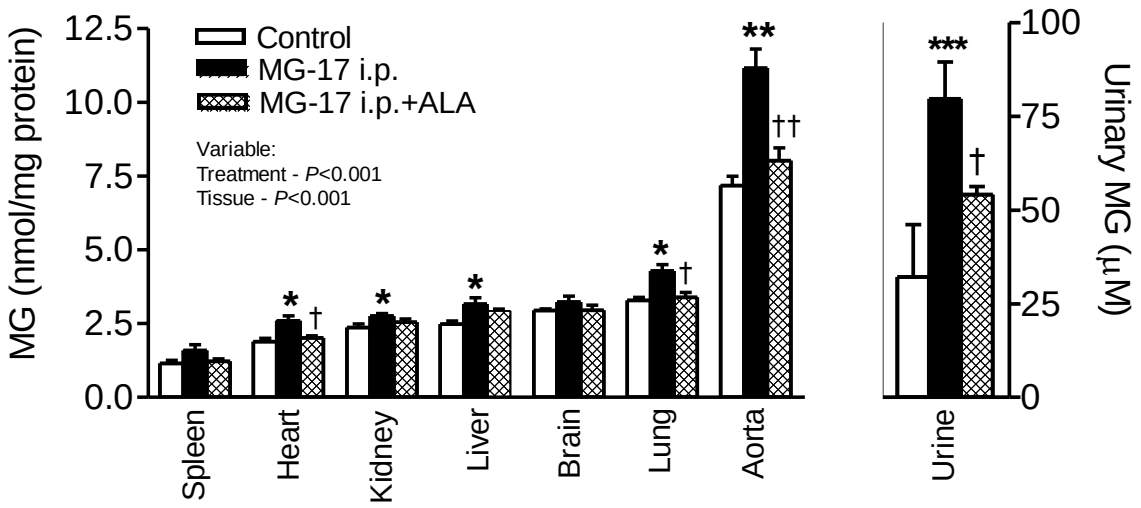


Figure 4. Distribution of methylglyoxal (MG) in different tissues and urine in Sprague-Dawley rats after intraperitoneal (17.25 mg/kg body wt) administration. [alagebrium (ALA; 100 mg/kg i.p.)]. **P*<0.05, ***P*<0.01, ****P*<0.001 *vs*. corresponding control group, †*P*<0.05, ††*P*<0.01 *vs*. MG-17 i.p. group. [Figure with permission from (Dhar et al, 2010a)].

Several studies have been done to calculate the levels of MG in animal tissues as well. The levels of MG in several different organs/tissues in normal male 12 week old Sprague-Dawley rats were measured in our lab to determine the basal levels and the levels of MG absorbed after intraperitoneal (i.p.) administration (Fig. 4) (Dhar et al, 2010a). The results revealed that the basal MG level in the aorta was apparently higher compared to other organs. The aortic MG level further increased significantly following i.p. administration. The urinary MG also increased significantly after i.p. administration indicating that most of the MG absorbed into the blood was being excreted in the urine.

Quantification of MG in ruminal fluid was attempted in one of the studies and a detection limit of 0.125μg/ml was calculated (Lodge-Ivey et al, 2004). Another very interesting study has

been conducted to calculate the levels of MG in various tissues of Sprague-Dawley rats using the electrospray ionization liquid chromatography mass chromatography (EI/LC/MC) technique. 5-Methylquinoxaline (5-MQ) is used as an internal standard. The results are expressed as mean value ± standard deviation (nmol/g wet weight). The values reported are: heart - 3.4±1.6, liver - 2.5±0.8, kidney - 2.0±1.0, aorta - 10.6±6.7 & blood - 2.0±1.0. Thus, the value of MG is highest in aorta followed by heart, liver, kidneys and blood (Randell et al, 2005). A Study has also been done to determine the extracellular & intracellular levels of MG in animal tissues. The intracellular concentration of MG expressed as mean± 2 standard deviations in Chinese Hamster cells grown in culture medium was in the range of 0.7±0.3μM to 1.2±0.3 μM while the extracellular concentration in the culture medium was in the range of 0.07±0.02 μM (Chaplen et al, 1996).

Glutathione

Reduced glutathione (GSH) is a major antioxidant in humans and other animals. Structurally it is a tripeptide synthesized from the amino acids glutamate, cysteine and glycine (Fig. 5). Glutathione reduces the disulfide bonds in proteins by acting as an electron donor. During this process, glutathione gets oxidized and is converted into glutathione disulfide (GSSG). The oxidized glutathione is converted back into the reduced form GSH by the enzyme glutathione reductase.

GSH also helps to remove the reactive hydrogen peroxide with the help of the enzyme glutathione peroxidase (Fig. 6). GSH levels in human tissues range between 0.1 and 10 mM.

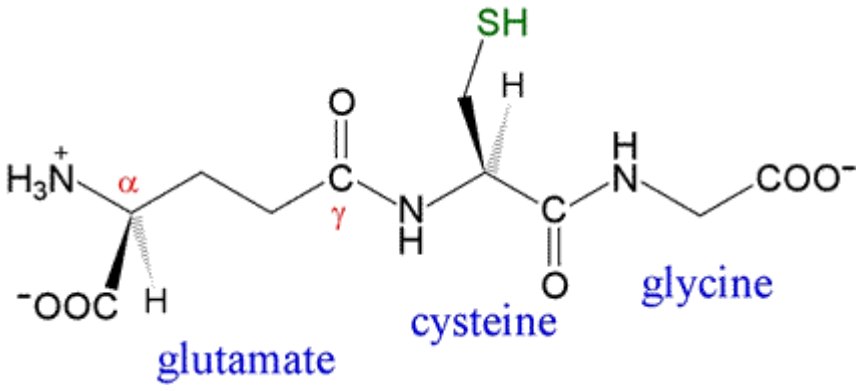


Figure 5. Structure of glutathione. (Source of image: http://guweb2.gonzaga.edu/faculty/cronk/biochem/G-index.cfm?definition=glutathione). Glutathione is synthesized from three amino acids, glutamate, cysteine and glycine.

The highest concentration is found in the liver (up to 10 mM), but the spleen, lens, kidney and blood cells (erythrocytes and leukocytes) also have high levels (Bremer, 1981). The plasma level of GSH is around 4.5 µM (Chawla et al, 1984). A number of conditions that increase oxidative stress can reduce GSH levels. These conditions include infections, toxic chemicals, inflammation (Thorne-Research-Inc., 2001) etc. Treatment with N-acetylcysteine (NAC), α-lipoic acid, methionine, ascorbic acid and taurine can increase GSH levels (Thorne-Research-Inc., 2001).

GSH plays a vital role in the degradation of MG by binding to free MG and presenting it to glyoxalase I which converts MG into hemithioacetal (Fig. 3).

Recent studies have shown that certain drugs, supplements and/or glutathione rich foods can be taken to increase and maintain optimum glutathione levels in plasma. Amongst these, calcitriol (an active metabolite of vitamin D_3), N-acetyl cysteine (NAC), S-adenosylmethionine (SAMe) and whey protein have been shown to be beneficial in raising glutathione levels.

Methylglyoxal and oxidative stress

MG is a double-edged sword because it is not only a potent inducer of oxidative stress itself but also leads to formation of advanced glycation end products (AGEs) which also cause oxidative stress (Desai & Wu, 2008; Wautier et al, 2001) (Fig. 6). So, it is a direct as well as indirect inducer of oxidative stress - a phenomenon wherein the levels of reactive oxygen species namely superoxide, hydrogen peroxide and peroxynitrite rise above the body's capacity to detoxify the reactive intermediates or repair any resulting damage. Incubating the cultured rat aortic vascular smooth muscle cells (VSMCs) with MG lead to an increased production of superoxide in a dose-dependent manner. MG also led to increased formation of hydrogen peroxide as well as peroxynitrite through induction of inducible NOS (iNOS) (Chang et al, 2005). Peroxynitrite can be formed by reaction of nitric oxide with excess superoxide which can lead to destruction of DNA and a wide variety of other molecules because of its oxidizing power (Pacher et al, 2007). Additionally, MG has also been shown to activate pro-oxidant p38 mitogen activated protein kinase (p38 MAPK) in human endothelial cells (Akhand et al, 2001). MG also increases NADPH oxidase mediated superoxide levels in rat kidney mesangial cells which was prevented by superoxide dismutase (Ho et al, 2007).

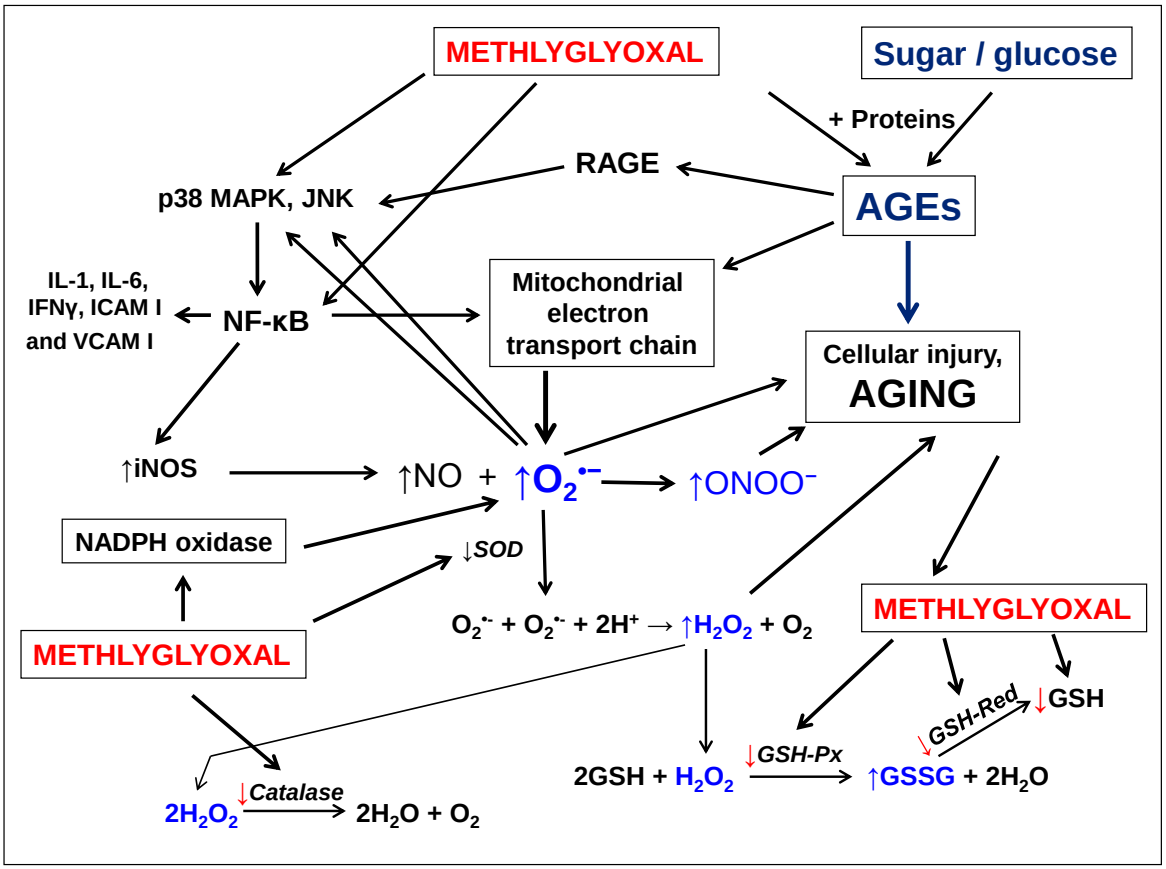


Figure 6. Mechanisms of methylglyoxal induced oxidative stress. [Image adapted from (Desai et al, 2010)].

The figure shows multiple mechanisms through which MG can induce or increase oxidative stress making it a very potent trigger for oxidative stress. MG has direct effects as well as indirect effects through the formation of advanced glycation end products (AGEs), which activate the receptor for AGEs (RAGE). MG and AGEs increase the expression and activity of nuclear factor kappa B (NF-κB), which in turn induces inducible nitric oxide synthase (iNOS). MG increases the expression and activity of NADPH oxidase and reduces the activity of catalase, glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red). AGEs and MG also increase superoxide ($O_2^{\bullet-}$) production from the electron transport chain. Abbreviations: H_2O_2, hydrogen peroxide; H_2O, water; ICAM-1, intercellular adhesion molecule 1; IFNγ,

interferon gamma; IL-1, interleukin 1; IL6, interleukin 6; JNK, c-Jun-N-terminal kinase; p38 MAPK, p38 mitogen activated protein kinase; $ONOO^-$, peroxynitrite; VCAM1, vascular cell adhesion molecule 1.

Apart from its pro-oxidant properties, MG also inhibits anti-oxidant mechanisms. MG interferes with anti-oxidant enzymes in blood cells. MG significantly reduces GSH levels in erythrocytes (Beard et al, 2003). As we have discussed above, MG is detoxified in the body using the glyoxalase system which is dependent on GSH levels. Moreover, MG inactivates glutathione peroxidase as well as glutathione reductase, which are the main enzymes responsible for the removal of hydrogen peroxide and recycling of GSH (Blakytny & Harding, 1992; Park et al, 2003). Since, MG decreases GSH levels, it impairs its self-destruction, increasing its half-life and thus, causes further oxidative damage through this positive feedback loop (Wu & Juurlink, 2002).

AGEs are the mediators for the indirect route of oxidative stress induction by MG. AGEs are produced as a result of interaction between MG and amino acid residues, mainly arginine, cysteine and lysine (Chang & Wu, 2006). AGEs produce oxidative stress by increasing levels of growth factors and cytokines (Westwood & Thornalley, 1996). Furthermore, AGEs can cause oxidative stress in cells like VSMCs, endothelial cells and mononuclear phagocytes by their interaction with the receptor for AGEs (RAGE) and other scavenger receptors (Thornalley, 1998; Wautier et al, 2001). Interaction of AGEs with RAGE leads to the expression of various cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) and also activates nuclear factor kappa B (NF-κB) which produces oxidative stress in the endothelial cells (Bierhaus et al, 1997; Kikuchi et al, 2003).

Oxidative stress further causes ageing and age-related diseases such as atherosclerosis and Alzheimer's disease (Baynes, 2001).

Methylglyoxal and advanced glycation end products formation

Advanced glycation end products or AGEs are a complex group of compounds formed by a non-enzymatic reaction between the reducing sugars and the amine group of proteins, lipids or nucleic acids (Goh & Cooper, 2008). They are formed from the reactive metabolic intermediates such as glucose, 3-deoxyglucosone and glyoxal. The reaction starts with the reducing sugar condensing with the amine group of protein at the N-terminal or on lysine side-chain. In the early phase of this reaction, the carbonyl group of sugar attaches with the protein to form an unstable intermediate called Schiff base (Desai & Wu, 2007). In the late phase of reaction, the Schiff base undergoes an Amadori re-arrangement to form a stable Amadori product. This Amadori product further gets cyclized to form a ring structure (Bucala & Cerami, 1992; Bucala et al, 1993). The Amadori product leads to the formation of various non-enzymatically derived products called AGEs by either oxidation, degradation or rearrangement. Oxidation of the glycated product is termed as Maillard reaction and owing to the brown product, it is also called browning reaction.

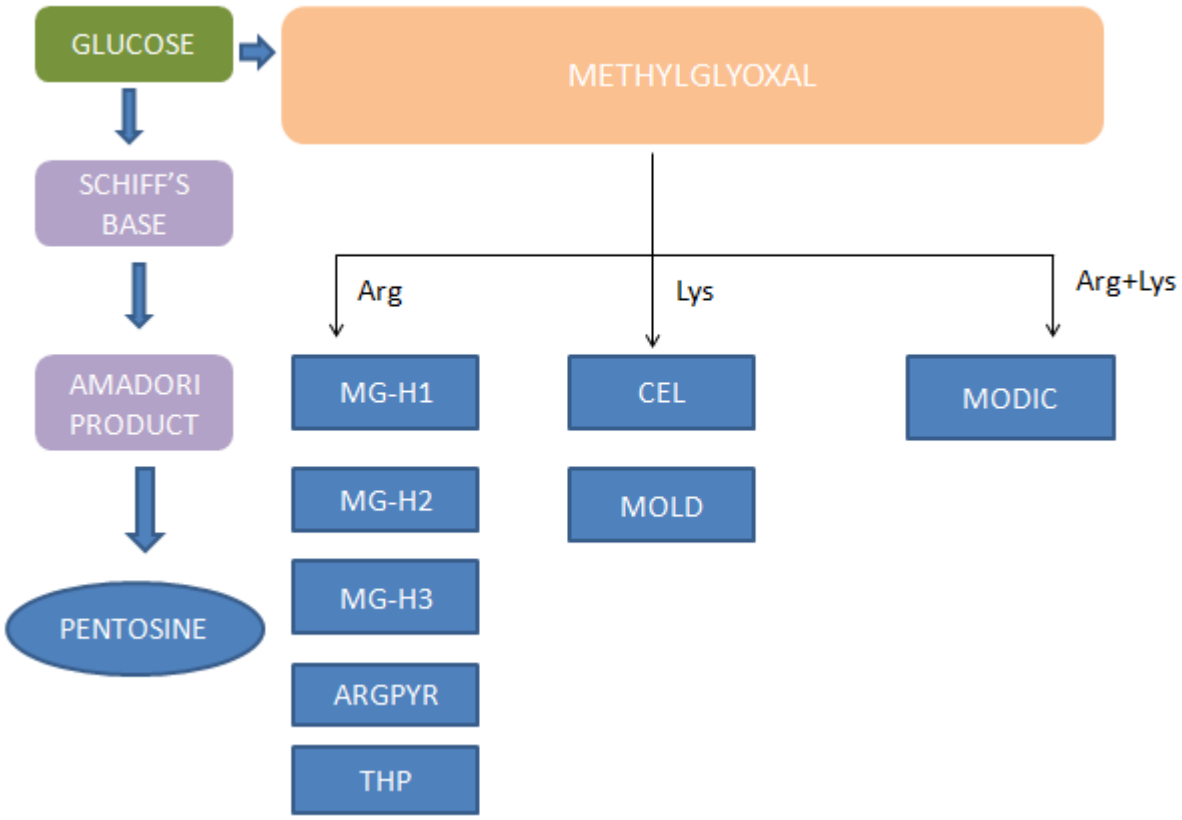


Figure 7. Different advanced glycation endproducts (AGEs) generated from methylglyoxal. [Image reproduced from (Matafome et al, 2013)].

The figure shows MG-derived AGEs. MG is a major source of AGEs. MG is mainly generated during glycolysis and from auto-oxidation of glucose. MG reacts with arginine (Arg) residues of proteins to form Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2), 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3), argpyrimidine (ARGPYR) and tetrahydropyrimidine (THP). It reacts with lysine (Lys) to form Nε-carboxyethylysine (CEL) and the methylglyoxal-lysine dimer (MOLD). A dimer crosslink between arginine and lysine results in methylglyoxal-derived imidazolium cross-linking (MODIC).

The major AGEs formed from MG can be broadly classified as fluorescent and non-fluorescent adducts (Fig. 7). MG reacts primarily with arginine producing cyclic imidazolone adducts. Depending upon the nitrogen atom involved and the environmental pH, three different products are possible. All these three adducts are in equilibrium since they can open up forming Carboxyethylarginine (CEA) (Klopfer et al, 2011). In addition, it can add another MG producing THP (tetrahydropyridimine) and through a final re-cyclization reaction yields argpyrimidine (Shipanova et al, 1997). MG also reacts with lysine residues to form carboxyethyllysine (CEL) (Fig. 7) whereas glyoxal forms carboxymethyllysine (CML). The aldimine intermediate further reacts with arginine guanidine group and through series of reactions yields a cross-linking imidazol MODIC adduct. MG can also react with two lysine residues forming another imidazolium MOLD adduct *via* a cannizaro-type reaction (Nasiri et al, 2011).

AGEs produce their biological effects by different and not so well-defined mechanisms (Vistoli et al, 2013). However, broadly speaking, AGEs exert their effects by two main mechanisms (Fig. 8). Firstly, they can directly cross-link with certain proteins such as collagen causing changes like vascular stiffness amounting to endothelial dysfunction. Secondly, they can cross-link with certain receptors such as RAGE and initiate a cascade of signaling reactions which cause release of various pro-inflammatory and pro-sclerotic agents (Goh & Cooper, 2008).

Besides RAGE some other receptors that AGEs generally bind to are AGE-receptors 1,2 and 3 or AGE-R1, AGE-R2 and AGE-R3 (McRobert et al, 2003) (Fig. 8). The interaction with RAGE triggers secondary messenger pathways like Protein-C whose major target is nuclear factor- κB. NF-κB ultimately leads to increased expression of proteins such as ICAM-1, E-selectin, vascular endothelial growth factor (VEGF), tissue factor and proinflammatory cytokines (Goldin et al, 2006; Schiekofer et al, 2003; Yan et al, 1994). AGE-R1 & R3 have been linked to

the clearance of AGEs and have been also implicated in diabetic complications due to AGEs (He et al, 2001).

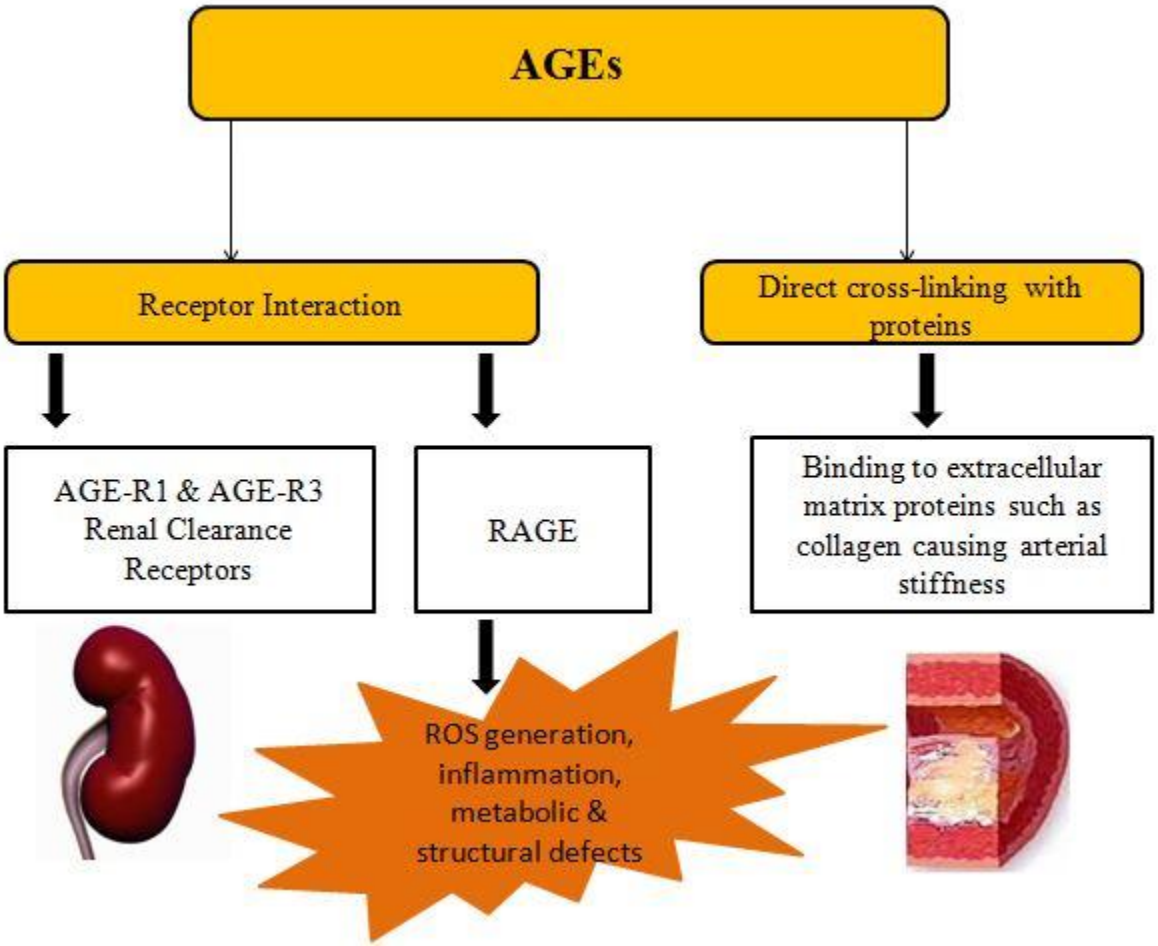


Figure 8. Mechanism of action of AGEs. [Image reproduced from (Forbes & Cooper, 2013)]. Abbreviations: AGEs, advanced glycation end products, AGE-R1, AGEs receptor 1; AGE-R3, AGEs receptor 3; RAGE, receptor for AGEs.

CML is the most commonly found AGE (Reddy et al, 1995). As already stated above, MG has a double-pronged mechanism of action. It induces oxidative stress independently and also indirectly by production of AGEs which further cause oxidative stress (Dhar et al, 2008). AGEs

have been found to accumulate in all the major tissues damaged by diabetes and the rate of accumulation is proportional to the level of hyperglycemia. The above described chain of events, especially vascular stiffness produced by AGEs, is found to have a role in causing the major diabetic complications in sites such as kidney, retina and atherosclerotic plaques (Bucala & Vlassara, 1995; Hammes et al, 1999; Makita et al, 1994).

Methylglyoxal toxicity and other pathological effects

The toxic effects of MG depend upon the dose and period of exposure. Over the years, various experiments have been performed *in vivo* and *in vitro* to study its toxic effects. Another set of studies brings out the pathological changes brought about in organisms upon prolonged exposure leading to different diseases.

In one such study, it was observed that a dose of 800 mg/body weight given intraperitoneally proved lethal to the experiments animals within 4 hours whereas animals injected with less than this lethal dose developed a reduced liver weight within 24 hours of treatment. Additionally, pulmonary hyperemia and distended intestines covered with fibrin were also observed (Choudhary et al, 1997; Kalapos et al, 1991b). In another study, MG given orally in female mice caused glomerular basement membrane thickening and collagen accumulation in kidneys which is one of the major renal changes in diabetes (Golej et al, 1998). Also, studies suggest that MG treatment also produces psychomotor changes in experimental animals. In case of rabbits, they developed ataxia, slow movements, depression and reduced appetite (Kalapos et al, 1991a).

On a broader scale, chronic accumulation of MG inside the organisms has been suggested as a causative agent in diseases such as cancer, beriberi, Alzheimer's disease, diabetes mellitus, atherosclerosis, hypertension and obesity.

Cancer

One such study suggested that the increased DNA synthesis and induced levels of ornithine decarboxylase (OCD) by MG suggest its carcinogenic potential (Furihata et al, 1985). In another study, MG was added to the drinking water of the animals for about 32 weeks and increased incidence of pyloric hyperplasia was observed (Takahashi et al, 1989). However, studies have also been conducted to suggest the opposite and hence, the role of MG in causing cancers is rather controversial.

Diabetes

MG is implicated in various micro and macro vascular complications of Diabetes Mellitus. Majority of these pathological changes are attributed to the phenomenon of AGEs formation and oxidative stress caused by MG. The accumulation of AGEs in kidneys leads to glomerulosclerosis, mesangial expansion, basement membrane thickening and tubulo-interstitial fibrosis and all these changes co-relate with structural aspects of diabetic nephropathy (Soulis-Liparota et al, 1995). Diabetic retinopathy mainly occurs by the loss of pericytes which is due to the thrombogenic and neovascularization activity of AGEs. This increases the permeability of proteins across the retinal barrier (Wautier & Schmidt, 2004). Diabetic neuropathy is characterized by nerve dysfunction and loss of pain perception and several studies proved the role of AGEs and RAGE in causing these changes by various mechanisms. One mechanism suggested that oxidative stress and NF-κB induce expression of certain pro-inflammatory genes

which cause loss of pain perception and neuronal damage (Sugimoto et al, 2008). This involves modification of axonal cytoskeleton mainly the proteins actin, tubulin and ECM protein laminin which leads to degeneration and demyelination of peripheral nerves (Huijberts et al, 2008).

Evidence also suggests the role of glycation, AGEs and AGEs/RAGE interactions in accelerated atherosclerosis and cardiovascular complications in Diabetic patients. AGEs lead to modification of LDLs (low density lipoproteins) which cause endothelial inflammation, increased permeability and transcytosis leading to local oxidative stress and LDL oxidation. This further leads to uptake of modified LDLs by scavenger-receptor pathways and finally foam cells and atheromatous plaques are formed (Chait et al, 1993; Sima et al, 2009; Tames et al, 1992). Also, AGEs lead to modification of certain ECM proteins especially collagen which lead to intima-media thickening and arterial stiffening (Bortolotto, 2007).

Hypertension

MG is also implicated in causing essential hypertension through the production of AGEs and reactive oxygen species (ROS) (Wu, 2006). AGEs are believed to cause hypertension mainly by decreasing the activity of the anti-oxidant enzymes and secondly by AGE-RAGE interaction leading to signaling of cellular pathways which ultimately lead to production of hypertension. One such pathway involves activation of $p21^{ras}$ followed by activation of MAPK leading to nuclear translocation of NF-κB (Bourajjaj et al, 2003; Schmidt et al, 1999). On the other hand, ROS cause hypertension by different mechanisms. MG leads to the production of superoxide, hydrogen peroxide and peroxynitrite ROS which lead to decrease in nitric oxide and decreased endothelium dependent vasodilation. It is said that excessive peroxynitrite formations is worse than diminished NO levels in causing hypertension (Zalba et al, 2001a; Zalba et al, 2001b).

Hydrogen peroxide is also an important intracellular signaling molecules which leads to activation of certain cellular pathways which produce hypertension by vascular remodeling, endothelial dysfunction & blood vessel wall inflammation (Escobales & Crespo, 2005; Irani, 2000; Wilcox, 2005). Several other mechanisms are also under investigation.

Alzheimer's disease

One suggested theory is through glycation of tau protein which leads to formation of neurofibrillary tangles, one of the hallmarks of Alzheimer's disease. Also, Amyloid-β peptide and β-sheet fibrils are ligands for RAGE which triggers oxidative stress, inflammation and amyloidosis amplifying the neurotoxic effects on microglia, blood-brain barrier and neurons (Chen et al, 2007). Additionally, various AGEs like CML, MOLD & GOLD have been found in senile plaques further suggesting the role of MG in causing Alzheimer's disease (Takeuchi & Yamagishi, 2008).

Obesity

Studies have proved that levels of MG adducts in diabetics correlate with the levels of LDL cholesterol and triglycerides. This suggests the possible role of MG in causing obesity (Turk et al, 2011). Studies have also demonstrated that increased MG levels cause structural and functional alterations in the adipose tissues leading to obesity (Matafome et al, 2012). Studies have also been done which prove the role of AGE/RAGE interactions in causing obesity (Leuner et al, 2012).

MG has been proven to play a role in causing certain other pathological processes such as impaired wound healing, ageing, osteoporosis and beriberi (Negre-Salvayre et al, 2009).

Scavengers of methylglyoxal

In the recent past, various agents have been studied which can scavenge MG directly or indirectly by inhibiting AGEs or modifying the pathways through which it exerts it deleterious effects. A few of those agents have been described below.

L-Arginine

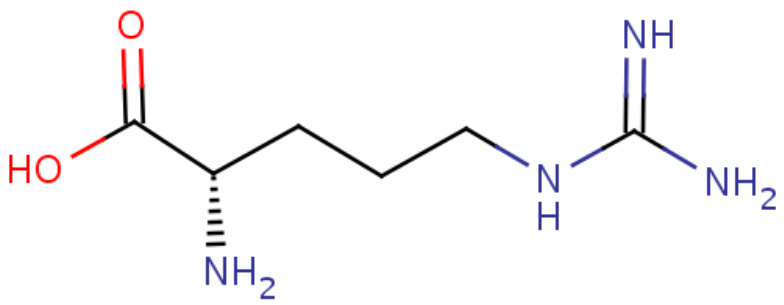


Figure 9. Structure of L-arginine (Source of image: http://www.drugbank.ca/drugs/DB00125)

L-arginine (Fig. 9) is a potent scavenger of MG which acts by e-NOS dependent and also through e-NOS independent mechanisms. L-arginine is a substrate for nitric oxide synthase (NOS) which leads to the production of nitric oxide (NO) and L-citrulline (Palmer et al, 1988). One of the important isoforms of NOS is endothelial NOS (eNOS) which mediates endothelium dependent vascular relaxation through production of NO. Reduced availability of eNOS can lead to diminished NO and hence, reduced endothelial vessel relaxation ultimately leading to endothelial dysfunction which is the hall mark of many diseases such as diabetes and hypertension (Potenza et al, 2009). Also, L-arginine is a substrate for another important enzyme i.e. arginase which is expressed mainly in the endothelium and is responsible for maintaining the eNOS as well as normal endothelial function. Hence, lack of this enzyme can also lead to

endothelial dysfunction (Berkowitz et al, 2003; Zhang et al, 2001). However, L-arginine can also attenuate MG and high glucose induced endothelial dysfunction, oxidative stress and AGEs by directly binding to MG and inactivating it (Dhar et al, 2012). Studies have shown that though L-arginine can attenuate the increased protein expression, it cannot control the increased arginase activity due to the high glucose diet. So, further studies need to be done to evaluate the protective role of L-arginine against MG before its therapeutic application.

Aminoguanidine

Aminoguanidine is the most extensively used MG and AGEs scavenger used till date. It acts by inhibiting the cross linking or glycation of the proteins and hence, prevents formation of AGEs from MG (Carvalho et al, 2011; Thornalley, 2003). Also, it inhibits the formation of MG from aminoacetone by irreversible inhibition of the enzyme SSAO which catalyzes this conversion (Casazza et al, 1984). Aminoguanidine can also reduce oxidative stress by inhibition of peroxynitrite and inducible NOS formation (Misko et al, 1993; Szabo et al, 1997). Studies have also proved that aminoguanidine prevents age related cardiac hypertrophy and aortic stiffening (Li et al, 1996). Also, it has been proved that aminoguanidine arrests age related hypertension in spontaneously hypertensive rats (SHR) by scavenging MG (Wang et al, 2007).

Alagebrium (ALT-711)

Alagebrium is mainly used as a AGEs cross-link breaking compound or an AGE- breaker. It is a stable derivative formed upon the degradation of phenacylthiazolium bromide (PTB) which is one of the first detected and commonly used AGE breaker (Wolffenbuttel et al, 1998). Studied have shown that intraperitoneal injection of ALT-711 (1 mg/kg) given daily for 1 or 3 weeks reversed diabetes induced arterial stiffening both *in vivo* and *in vitro* (Zieman et al, 2007). It is

the only AGEs cross breaker being tested in human trials. A clinical study conducted by nine US centers has found that aged patients treated with ALT-711 showed improved arterial compliance and reduced vascular stiffening (Kass et al, 2001). Hence, ALT-711 can be a very useful therapeutic weapon against AGEs. Another set of studies have shown acute effects of alagebrium on MG and changes produced by it. It was seen that alagebrium attenuated the increased MG levels in the plasma, aorta, heart, kidney, liver, lung and urine after MG administration. In MG-treated rats, glucose tolerance was impaired, plasma insulin levels were higher and insulin-stimulated glucose uptake by adipose tissue was reduced and all these changes were reduced by treatment with alagebrium (Dhar et al, 2010a).

Metformin

Metformin is an oral hypoglycemic agent which has a guanidino structure and this means it can not only lower the blood glucose levels but also react with α-dicarbonyl compounds and prevent their toxic effects as well as inhibits the formation of AGEs (Beisswenger et al, 1999). In this study, it was suggested that metformin reduces MG levels in a dose dependent manner. Another possible mechanism of metformin action is that it increases levels of reduced glutathione and hence, enhances detoxification of MG (Rahbar et al, 2000). It is also a potent inhibitor of glycation. Chronic treatment with metformin lead to a reduction in AGEs formation in lens, kidneys and various other organs in diabetic rats (Tanaka et al, 1999).

N-acetyl cysteine

N-acetyl cysteine (NAC) is another potent MG scavenger (Jia & Wu, 2007; Vasdev et al, 1998) which is a cysteine containing compound. Since, MG has a high affinity to cysteine, it

readily binds with NAC. Also, N-acetyl cysteine can increase levels of GSH which leads to increased degradation of MG (McLellan et al, 1994). It can also directly react with free radicals and is thus, a potent antioxidant as well (Aruoma et al, 1989; Dekhuijzen, 2004).

Other than MG scavengers, there are agents which act specifically on AGEs and we divide these into 2 categories: (i) AGE inhibitors & (ii) AGE breakers.

AGE inhibitors are defined as agents, which target one of the steps in the synthesis of MG. On the other hand, AGE breakers break cross-linking between proteins and hence, degrade AGEs. Table 1 below shows a few members of both these classes.

AGE BREAKERS	AGE INHIBITORS
Phenacylthiazolium bromide (PTB)	Aminoguanidine
Alagebrium (previously ALT-711)	Metformin
5-aminosalicylic acid (5-ASA)	Pioglitazone
	Tenilsetam

Table 1. AGE inhibitors and breakers

High carbohydrate diets

In terms of biochemistry, carbohydrates are macromolecules consisting of carbon, hydrogen and oxygen atoms. Another term used for them is 'saccharide' and they are classified into 4 different groups, namely: (i) monosaccharides (ii) disaccharides (iii) oligosaccharides and (iv) polysaccharides. Generally, mono and disaccharides are smaller carbohydrate molecules and are known commonly as 'sugars'. Oligosaccharides usually are polymers of monosaccharides (3-9)

and found mainly on the plasma membrane of animal cells. Polysaccharides are very big and bulky polymers formed by long chains of monosaccharide units combined by glycosidic bonds (Flitsch & Ulijn, 2003). Examples of monosaccharides are glucose and fructose whereas sucrose (glucose + fructose) and lactose (glucose+galactose) are examples of disaccharides. Fructo-oligosaccharides are an examples of the third family (Bode, 2009). Cellulose, starch and glycogen are examples of the polysaccharides.

High carbohydrate diets are traditionally defined as foods with high glycemic index (71-100) but now the view is changing. Sugars such as fructose which has a lower glycemic index has become a major concern owing to its increased rates of consumption in the modern day diet and its association with various pathological conditions such as obesity, dyslipidemia, diabetes, hypertension and insulin resistance (Singh et al, 2008). Fructose is found in the soft drinks, sweetened beverages and high fructose corn syrup (HFCS) (Table 2). Since sucrose gets degraded to fructose and glucose in body, consumption of sucrose leads to increased levels of these sugars in the blood and hence, sucrose rich diets are also under study because of the increasing view that dietary patterns especially the increased sugar consumption rates are connected with the pathogenesis of several diseases.

Year	HFCS	Total caloric sweeteners	HFCS as percentage of total caloric sweeteners	Percentage of HFCS from HFCS-42	Percentage of HFCS from HFCS-55
	$g \cdot person^{-1} \cdot d^{-1}$	$g \cdot person^{-1} \cdot d^{-1}$	%	%	%
1966	0.0	165.9	0.0	—	—
1970	0.8	175.1	0.4	100.0	0.0
1975	7.1	168.8	4.2	100.0	0.0
1980	27.3	176.0	15.5	71.2	28.8
1985	64.7	184.4	35.1	34.3	65.7
1990	71.0	195.7	36.3	41.0	59.0
1995	82.3	211.7	38.9	39.9	60.1
2000	91.6	218.0	42.0	38.8	61.2

Table 2. Availability of high fructose corn syrup (HFCS) in the caloric sweetener supply in United States. HFCS-42 and HFCS-55 contain 42% and 55% fructose, respectively. [Source of table: (Bray et al, 2004)].

Various studies have been done to prove the deleterious and harmful effects of high carbohydrate diets. In mice, it was seen that treatment with high carbohydrate diets lead to metabolic dysfunction by increasing visceral adiposity and glucose intolerance and altering levels of leptin and adiponectin. It also causes distortions in regulation of body weight and predisposes them to early adult-onset obesity (Srinivasan et al, 2008). Meta-analysis of clinical trials with high carbohydrate diets has proven that they are associated with increased cardiovascular risks, greater magnitude of insulin resistance and poorer lipid profiles in type-2 diabetes mellitus patients (Kodama et al, 2009).

Differences between Fructose, Sucrose and Glucose

Structure

While glucose and fructose have the same molecular formula, their chemical structures are different. Glucose forms a pyranose ring structure (six-membered ring) and fructose has a

furanose ring structure (five-membered ring) (Fig. 10). They both condense to form a disaccharide sugar called Sucrose. Their structures can be represented as shown below:

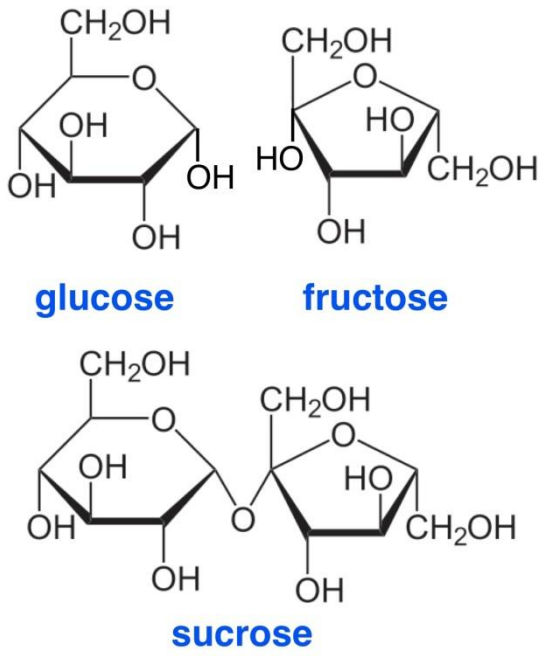


Figure 10. Chemical structures of all glucose, fructose and sucrose.

Image-source: http://www.themadscienceblog.com/2013/08/sugars-sugar-alcohols-and-sweet-taste.html

Absorption and metabolism

Disaccharides such as sucrose are cleaved into glucose and fructose on entering the intestine. Glucose and fructose are absorbed and metabolized differently in the body. Looking at the mechanisms of their absorption, glucose is absorbed actively by a sodium dependent transporter, glucose transporter 4 (GLUT4) which is located on the apical membrane of the intestinal epithelial cells. On the other hand, fructose is absorbed down duodenum and jejunum

by facilitated diffusion through another transporter, GLUT 5 (Bray et al, 2004). This process does not require energy being non-sodium dependent (Havel, 2005).

Also, these two sugars differ in the way they are metabolized in the body. Glucose metabolism is tightly regulated by insulin and phosphofructokinase. Glucose triggers the release of insulin from the pancreatic beta cells and in turn, insulin signals the insulin sensitive tissues i.e. muscle cells, adipocytes and liver cells to take up and store glucose. Insulin also regulates the GLUT 4 expression (Czech, 1995). When insulin levels are low, GLUT4 glucose transporters are present inside the cytoplasmic vesicles where they are used for transporting glucose. Binding of the insulin to receptors on such cells signals these tissues and leads to the rapid fusion of these vesicles with the plasma membrane and insertion of the glucose transporters, thus, giving the cells the ability to take up glucose. Again, when the insulin concentration in blood falls, these transporters are recycled back into the cytoplasm. Hence, glucose is mainly metabolized in the extra-hepatic tissues.

In contrast to this, fructose is mainly metabolized in the liver itself and the process is non-insulin dependent. Fructose metabolism bypasses phosphofructokinase and fructose is phosphorylated to fructose-1-phosphate by fructokinase and ATP. Hence, chronic fructose consumption can up-regulate fructokinase enzyme activity in rats and humans & can lead to ATP depletion (Ouyang et al, 2008).

Lipogenesis

Fructose is more lipogenic than glucose. Fructose-1-phosphate generated by fructose phosphorylation is further catalyzed by aldolase B to glyceraldehyde (GA) & dihydroxyacetone (DHA) phosphate which are intermediated in the glycolysis pathway. DHA phosphate can be converted into glycerol-3-phosphate which provides the glycerol portion for the triglyceride

synthesis (Havel, 2005). GA also proceeds to generate pyruvate which further forms the acetyl-coA catalyzed by pyruvate dehydrogenase, hence, providing substrate for fatty acid synthesis. Thus, by provision of both glycerol-3-phosphate as well as substrates for fatty acid synthesis, fructose triggers the de novo synthesis of triglycerides (Mayes, 1993). Fructose also leads to decreased very low density lipoproteins (VLDL)-triacylglycerol clearance (Faeh et al, 2005).

Also, unlike glucose, fructose does not stimulate leptin secretion (Bray et al, 2004) due to lack of GLUT 5 receptors in the pancreatic beta cells. Leptin promotes the feeling of satiety and lack of leptin secretion with a fructose rich diet prolongs hunger and enhanced desire for food which leads to increased body fat. This can lead to increased obesity in people consuming fructose rich diets.

Despite all their differences, there are however some similarities too between glucose and fructose. Both of them are associated with production of MG (Fig. 11) as well as generation of reactive oxygen species and causation of oxidative stress. Also, both of them are capable of producing the AGEs (Beisswenger et al, 2003; Rosca et al, 2005). Both of them have been implicated in several diabetic complications and worsening of the diabetic state in general. Additionally, fructose also leads to metabolic syndrome (Rutledge & Adeli, 2007) and other chronic diseases such as fatty liver and kidney toxicity (Gaby, 2005).

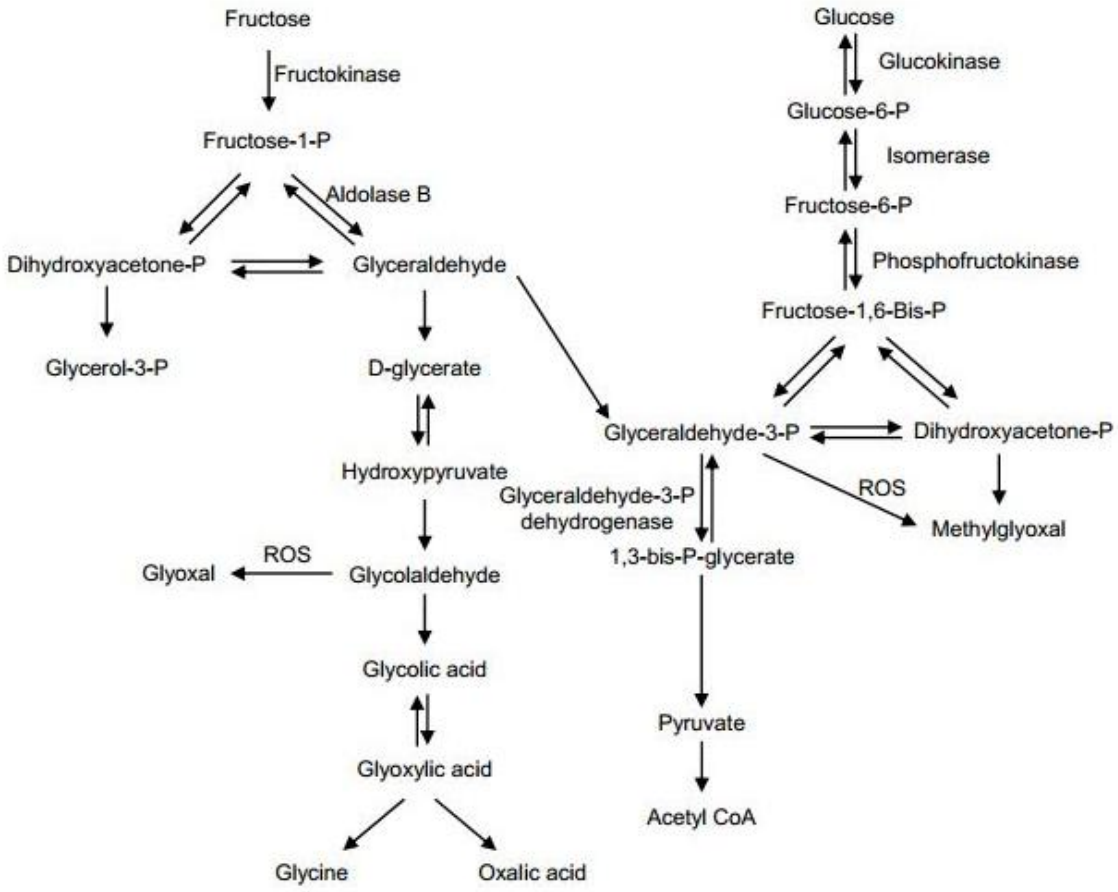


Figure 11. Generation of methylglyoxal from fructose and glucose. The figure shows the metabolic pathway of fructose, which bypasses phosphofructokinase, a regulatory enzyme for the glycolysis pathway. This means that when fructose is metabolized there is a continuous supply of substrates to the glycolysis pathway and lipogenesis. Both pathways provide triose phosphate intermediates from which methylglyoxal is formed. Abbreviations: Fructose-1-P, fructose-1-phosphate; Glycerol-3-P, glycerol-3-phosphate; P, phosphate; ROS, reactive oxygen species. [Source of figure: (Danpure, 2004)].

My project was designed with a primary aim to increase our knowledge about the endogenous generation of MG from two of the most commonly, encountered monosaccharides in our diet, *viz*. glucose and fructose. The detailed rationale for my project is described in the following section.

Rationale for the study

MG has been known to scientists for many years. Numerous research articles have been published. Yet, the surprising fact is that not enough is known about the endogenous production of MG in different organs and cells. For example the endogenous levels of MG in all the organs and tissues are not known. The plasma concentrations of MG have been measured and reported in several studies but only in limited species such as humans, rats and mice (Espinosa-Mansilla et al, 2007; Espinosa-Mansilla et al, 1998; Haik et al, 1994; Jia & Wu, 2007; Kandhro et al, 2008; Lodge-Ivey et al, 2004; Nemet et al, 2004; Wang et al, 2007). Moreover, the concentrations reported in a given species vary quite a bit, which most probably is due to the method employed. MG is reactive and unstable and mostly exists in a bound form (Chaplen et al, 1998). It is well established that glucose is the most abundant precursor of MG formed metabolically in the body. This fact assumes great importance currently because our diets have very high amounts of carbohydrates, especially free glucose, fructose and sucrose. Theoretically, consumption of high amounts of these monosaccharides and disaccharide would produce high amounts of MG. Also, it is a well-known biochemical fact that glucose and fructose are metabolized differently in the body (Johnson et al, 2009; Johnson et al, 2007; Wolfe & Ahuja, 1977). Therefore, our aim for this study was to determine MG levels in the plasma, urine and different organs/tissues of Sprague-Dawley rats fed chronically with a high glucose or a high fructose diet. We chose a commercial source (Harlan Laboratories Inc., Madison, WI, USA) of these diets to standardize the feed. The diets had either high fructose or high glucose, which provided 60% of total daily caloric requirements. The average North American diet is believed to provide upto 15% of total calories from fructose (Vos et al, 2008), thus the 60% carbohydrate diets we chose provides higher amounts of calories from carbohydrates. However, this can be

justified by the fact that the commonly used normal rat chow provides 60% of total calories from carbohydrates, mainly in the form of starch (LabDiet.com, 2013). Moreover, a high fructose diet providing 60% of total caloric intake is typically used for research in animals (de Moura et al, 2009; Galipeau et al, 2001; Oudot et al, 2013; Sanchez-Lozada et al, 2010; Tobey et al, 1982; Zavaroni et al, 1980). The idea is that using 60% of carbohydrate derived calories will speed up any carbohydrate-induced pathology to facilitate a shorter feeding time and a more practical research protocol.

Any observed pathological effect believed to be due to MG can be confirmed by preventing or attenuating it with a MG scavenger. Arginine has great affinity for MG (Lo et al, 1994; Takahashi, 1977) and our lab has shown it to be an effective MG scavenger *in vitro* (Dhar et al, 2012). However, there are hardly any papers showing that arginine is an effective MG scavenger *in vivo*. Therefore, we decided to use L-arginine as a MG scavenger for our *in vivo* study. If L-arginine is shown to be an effective MG scavenger *in vivo* then it can be safely used to prevent the harmful effects of high carbohydrate diets that are mediated by excess MG production. L-arginine is already used as an oral supplement by people for various conditions (Alvares et al, 2011; Bailey et al, 2010; Clarkson et al, 1996; Coman et al, 2008; Creager et al, 1992; Dong et al, 2011; Koga et al, 2006). The fact that L-arginine can enhance NO production would be an added benefit to the MG scavenging effect of L-arginine.

Hypotheses and objectives

Hypotheses:

1. High fructose and high glucose diets produce different amounts of methylglyoxal in the plasma and different organs/tissues of the body.
2. The GSH levels in different organs/tissues will be reciprocal to the methylglyoxal levels.
3. Glucose tolerance to an oral glucose load is associated with methylglyoxal levels in the plasma, pancreas, adipose tissue, liver and skeletal muscle.
4. L-arginine will attenuate the increase in methylglyoxal levels caused by high carbohydrate diets in the plasma and different organs/tissues by acting as a scavenger.

Objectives

1. To investigate whether chronic feeding for 12 weeks with a high glucose or a fructose diet produces different amounts/levels of MG in the plasma and different organs/tissues in Sprague-Dawley rats.
2. To measure and compare the amounts of reduced glutathione (GSH) following chronic high fructose or high glucose diet feeding in different organs of the body and determine differences and similarities.
3. To examine the effects of a high glucose and a high fructose diet on oral glucose tolerance and the insulin response in the plasma.
4. To examine whether L-arginine can attenuate or prevent any elevations of MG levels in the plasma or different organs/tissues in rats.

Methods and materials

Animals

All animal protocols were approved by the University of Saskatchewan's Animal Research Ethics Board, following guidelines of the Canadian Council on Animal Care. Male 9-week old 45 Sprague-Dawley rats (Charles River Laboratories) were used after one week of acclimatization. The following treatments were used: (i) **Carbohydrate (Carb) control diet**: Cat # **TD.05075** (Harlan Laboratories Inc., Madison, WI, USA), (ii) **High fructose diet**: **Teklad Cat # TD.89247, 60% fructose diet**, (iii) **High glucose diet**: **Teklad Cat # TD.05256, 60% glucose diet**, (iv) **L-arginine: 500 mg/kg/day in drinking water** (Lee et al, 2009; Matsuoka et al, 1996; Xu et al, 2010) (pH adjusted to 7.4). L-arginine was given in drinking water due to the long period of administration spanning 12 weeks. Administration of drugs by daily gavage for 84 days would have caused a lot of stress and would most likely not be approved by the Animal Ethics Board due to the alternate available option of giving it in drinking water. The rats were randomly divided into the following treatment groups (n = 6-8 each):

(1) Control – Normal chow diet (n = 6), **(2) High fructose** (60% Fructose diet) (n = 8), **(3) High fructose** (60% in diet) + **L-arginine** (a methylglyoxal scavenger, 500 mg/kg/day in drinking water) (n = 8), **(4) High glucose** (60% glucose diet) (n = 8), **(5) High glucose** (60% in diet) + **L-arginine** (500 mg/kg/day in drinking water) (n = 8), **(6) L-arginine** (500 mg/kg/day in drinking water) alone (n = 7). All groups were treated for 12 weeks (we have treated rats with a high sucrose diet, but the samples have not been analyzed).

Body weight was recorded before and at the end of the treatment period since high carbohydrate diets are lipogenic (Samuel, 2011). Mean arterial pressure (MAP) was measured at the end of the 12 week treatment period, since high fructose (Chen et al, 2010; Hwang et al, 1987; Jalal et al, 2010; Madero et al, 2011a; Madero et al, 2011b; Perez-Pozo et al, 2010) and high glucose (Kaufman et al, 1991) have been reported to increase the blood pressure (BP). Fructose-induced hypertension has been ascribed to an increase in synthesis and plasma and intracellular levels of uric acid (Madero et al, 2011b; Perez-Pozo et al, 2010). Since changes in the BP were not the primary focus of this study, we did not use radiotelemetry to measure the BP, which is invasive and may cause inflammation, stress, and risk of infection. After the 12 week treatment period the rats were fasted overnight (12-16 h with free access to water only). The MAP was measured over 30 min with an intrarterial (right carotid artery) catheter in the anesthetized (thiopental sodium 100 mg/kg i.p.) rat (Laight et al, 1999), following which a basal blood sample (0.5 mL) was collected in an EDTA tube and the plasma was separated and stored at -80° C to measure levels of MG (by HPLC), fasting glucose and insulin levels. Freshly separated plasma from 1 mL blood collected in a heparinized tube was sent to the Clinical Biochemistry lab at the Royal University Hospital to measure triglycerides, cholesterol and high density lipoprotein (because high carbohydrate diets can alter lipid levels) levels. An oral glucose tolerance test was performed as described later (Dhar et al, 2011). The rat was killed by cutting open the heart at the end of the experiment and causing exsanguination. The organs/tissues were quickly removed, rinsed in 0.9% saline, frozen in liquid nitrogen and stored at -80º C.

Measurement of mean arterial pressure

The mean arterial pressure (MAP) was measured at the end of 12 weeks of treatment with an intra-arterial catheter in anesthetized rats, a technique routinely used in our lab (Desai et al, 2006). The rats were anesthetized with an intraperitoneal injection of thiopental sodium (100 mg/kg body wt in a 25 mg/ml solution), and placed on a heated pad to maintain a temperature of 37°C. The trachea was cannulated with a PE240 tubing to facilitate spontaneous respiration. The right carotid artery was cannulated with a PE50 tubing. The carotid cannula (id 0.5, od 1.0 mm) was filled with heparinized saline (50 U/ml) and connected to a pressure transducer to record mean arterial pressure using the Powerlab data acquisition system and the Lab Chart Pro software (AD Instruments Pvt. Ltd., Sydney, Australia).

Measurement of methylglyoxal

MG was measured by a specific and sensitive high performance liquid chromatography (HPLC**)** method which was standardized in our lab (Dhar et al, 2009). The frozen organs were powdered under liquid nitrogen. A weighed amount of organ/tissue powder was reconstituted in sodium phosphate buffer (pH 4.5), vortexed and kept on ice. The sample was sonicated (30 s pulses, three times). The sample was then centrifuged (12,000 rpm for 10 min at 4° C). The supernatant was removed and kept on ice for further analysis. The pellet was discarded. The supernatant was incubated with 0.45 N (final concentration) perchloric acid (PCA) and 10 mM *o*-phenylenediamine (*o*-PD) for 24 h in a closed box at room temperature to protect it from light. The PCA incubation was performed to precipitate all the soluble proteins and also to free the reversibly bound MG from proteins and other molecules. PCA would also inhibit metabolic

reactions. *o*-PD was used as a thermodynamic trap to bind and derivatize the free MG and form the stable product 2-methylquinoxaline (2-MQ) (Fig. 12) (Dhar et al, 2009).

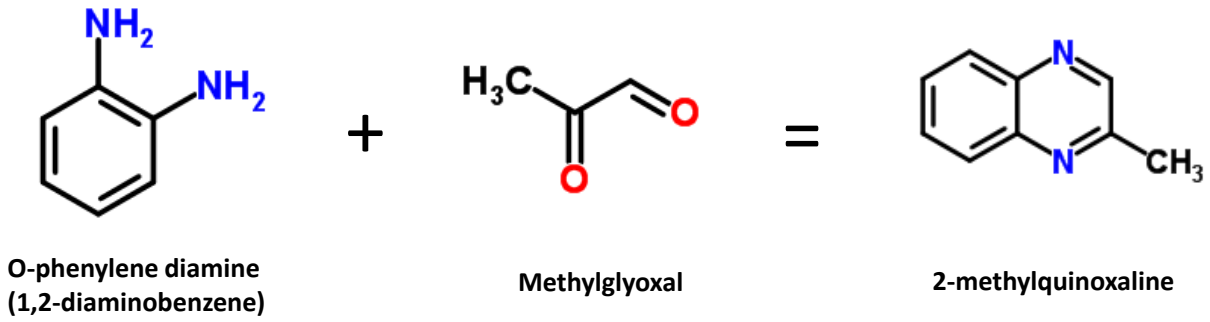


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Figure 12. Derivatization of methylglyoxal for HPLC. Free methylglyoxal in the sample was derivatized with o-phenylene diamine to form the stable 2-methylquinoxaline, which was then quantitated with high performance liquid chromatography.

Next day the samples were centrifuged at 12000 rpm for 10 min. The supernatant was mixed with the internal standard 5-methylquinoxaline (10 µM final concentration, 10% vol: vol). The 2-methylquinoxaline and 5-methylquinoxaline internal standard were detected with a UV detector on a Hitachi D-7000 HPLC system (Hitachi Ltd., Mississauga, ON, Canada), with a Phenomenex kinetex C18 (5 µm, 100 Å) column (150 x 4.6 mm). 20% acetonitrile and 8% sodium phosphate (pH 4.5) in distilled water were used to constitute the mobile phase. All samples were analyzed in duplicates. The ratio of the area under the curves for 2-MQ and 5-MQ peaks was used to calculate the MG concentration from a standard curve of known 2-MQ concentrations. The concentration of MG in organ/tissue samples was standardized to the protein concentration of the sample.

The protein concentration of the supernatant obtained after sonication and centrifugation was determined using a Bio-Rad *DC* protein assay kit (Cat # 500-0111, Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada),

Measurement of reduced glutathione: Monochlorobimane (mCB) approach

The GSH in a sample was measured fluorometric method using monochlorobimane (mCB), which was standardized by Kamencic et al (Kamencic et al, 2000). The working principle of this method is that mCB added to a sample of tissue homogenate reacts with GSH to form a fluorescent GSH-mCB adduct. This reaction is catalyzed by glutathione-S-transferase.

For this assay the frozen organs were powdered under liquid nitrogen. A weighed amount of organ/tissue powder was reconstituted in 20 vol of cold 50 mM Tris buffer (pH 7.4), vortexed and kept on ice. The sample was sonicated (30 s pulses, three times). The sample was then centrifuged (12,000 rpm for 10 min at 4° C). The supernatant was removed and kept on ice for further analysis. The pellet was discarded. mCB was added to the supernatant to a final concentration of 100 µM along with glutathione *S*-transferase (1 U/ml, Cat. # G 6511, Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada) obtained from equine liver. The homogenate was then allowed to incubate at room temperature for 30 min. The GSH–mCB adduct was measured in a Labsystems Fluoroskan II microtiter reader with excitation at 380 nm and emission measured at 470 nm.

GSH standards preparation

To prepare the standards, GSH was dissolved in 50 mM Tris buffer (pH 7.4) to obtain a final concentration of 1.0 mM. This GSH standard solution was incubated with mCB and glutathione *S*-transferase. GSH and glutathione *S*-transferase (Cat. No. G 6511) were purchased from Sigma (Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada). Monochlorobimane (Cat # M-1381MP) was purchased from Life Technologies (Life Technologies Inc., Burlington, ON, Canada).

Oral glucose tolerance test

An oral glucose tolerance test was performed as described earlier by our lab (Dhar et al, 2011). The rat was fasted overnight and placed in a metabolic cage. The urine was collected. The rat had free access to plain water or L-arginine (500 mg/kg body wt) containing water as the case may be. After the overnight fast the rat was anesthetized with an intraperitoneal injection of thiopental sodium (100 mg/kg body wt in a 25 mg/ml solution), and placed on a heated pad to maintain a temperature of 37°C. The trachea was cannulated with a PE240 tubing to facilitate spontaneous respiration. The right carotid artery was cannulated with a PE50 tubing.

After a basal blood sample of 0.5 mL was collected in an EDTA containing tube from the carotid artery, an oral glucose load (1 g/kg body wt) was given with a stomach tube. Further blood samples of 0.4 mL each were collected at 15, 30, 60, and 120 min from the carotid artery. The samples were always kept on ice. The blood sample containing Eppendorf tubes were centrifuged at 12,000 rpm for 10 min and the plasma was separated and stored at -80° C for later analysis. Plasma glucose levels were determined using a glucose colorimetric assay kit (Cat # 10009582, Cayman Chemical Co., Ann Arbor, MI, USA). The insulin levels were measured with a rat insulin ELISA assay kit (Cat # 10-1250-01, Mercodia Inc., Winston Salem, NC, USA). The

results were plotted using the PRISM software (v. 5, GraphPad Software Inc., La Jolla, CA, USA) and analyzed statistically.

Statistical analysis

Statistical analysis of results was performed with one way analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine significant differences between pairs of all groups. A *P* value of less than 0.05 was considered to be significant. The results are expressed as mean ± standard error of mean (SEM).

RESULTS

High glucose and high fructose diets significantly increase the mean arterial pressure in rats but did not affect the body weight

Feeding 10 week old male Sprague-Dawley rats for 12 weeks with either a high glucose (60% of total calories) or a high fructose (60% of total calories) diet did not affect the increase in body wts compared to the control group (Fig. 13). However, the high glucose and high fructose diets significantly increased the mean arterial pressure, compared to the control (Fig. 14), as measured with a carotid artery cannula in anesthetized rats at the end of the 12 week treatment period. Co-treatment with the MG scavenger L-arginine (500 mg/kg body wt in drinking water) did not attenuate the increase in mean arterial pressure caused by high glucose and high fructose diets. Surprisingly, treatment with L-arginine alone also caused a significant increase the mean arterial pressure compared to the control (Fig. 14).

High fructose diet significantly increases plasma total cholesterol and triglyceride levels in rats

Treatment of 10 week old male Sprague-Dawley rats for 12 weeks with a high fructose diet significantly increased the fasting plasma levels of total cholesterol and triglycerides compared to the control group (Figs. 15A, D). Co-treatment with L-arginine attenuated fructose-induced increase in total cholesterol but not in triglyceride level (Figs. 15A, D). Treatment with a high glucose diet for 12 weeks did not increase plasma total cholesterol or triglyceride levels compared to the control (Figs. 15A, D). L-arginine alone also did not increase plasma total cholesterol or triglyceride levels compared to the control group. There was no change in HDL levels or the total cholesterol/HDL ratio with either of the treatments (Fig. 15B, C).

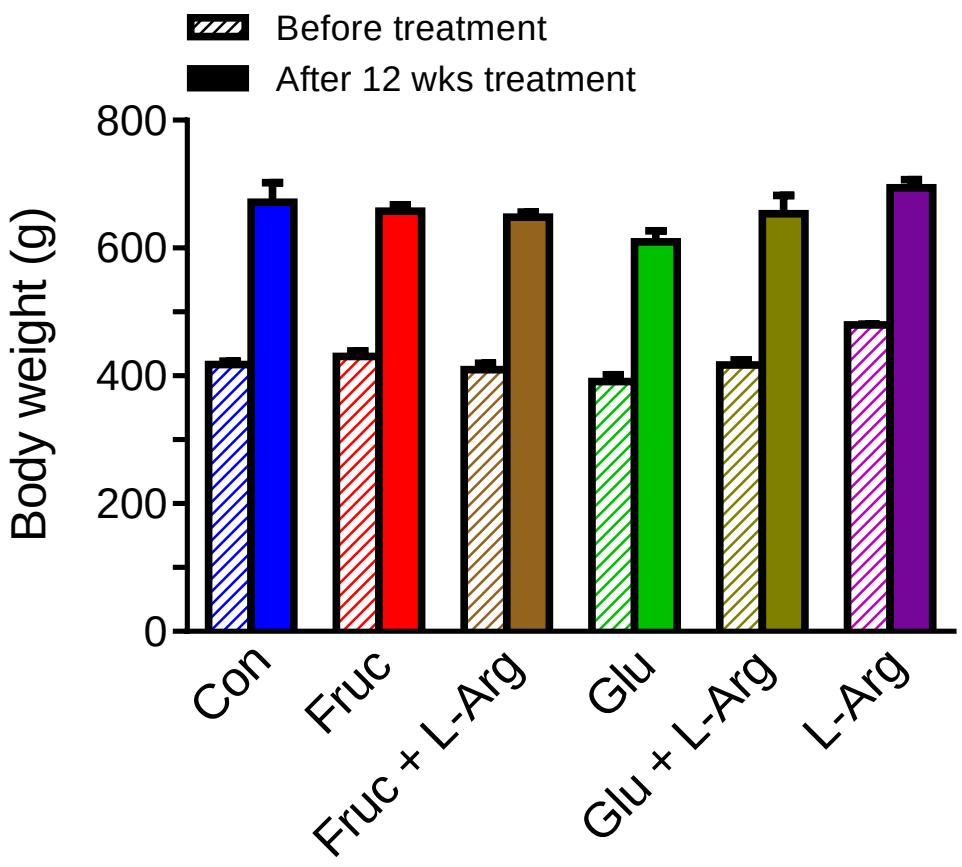

Figure 13. High glucose and high fructose diets do not affect the increase in body weights, compared to control, in rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg, 500 mg/kg body weight in drinking water) for 12 weeks. The body weights were measured at the start of treatment (10 weeks, hatched bars) and at the end of 12 weeks of treatment (solid bars). Statistical analysis was performed with a one way ANOVA followed by Tukey's post-hoc test. (n = 6-8).

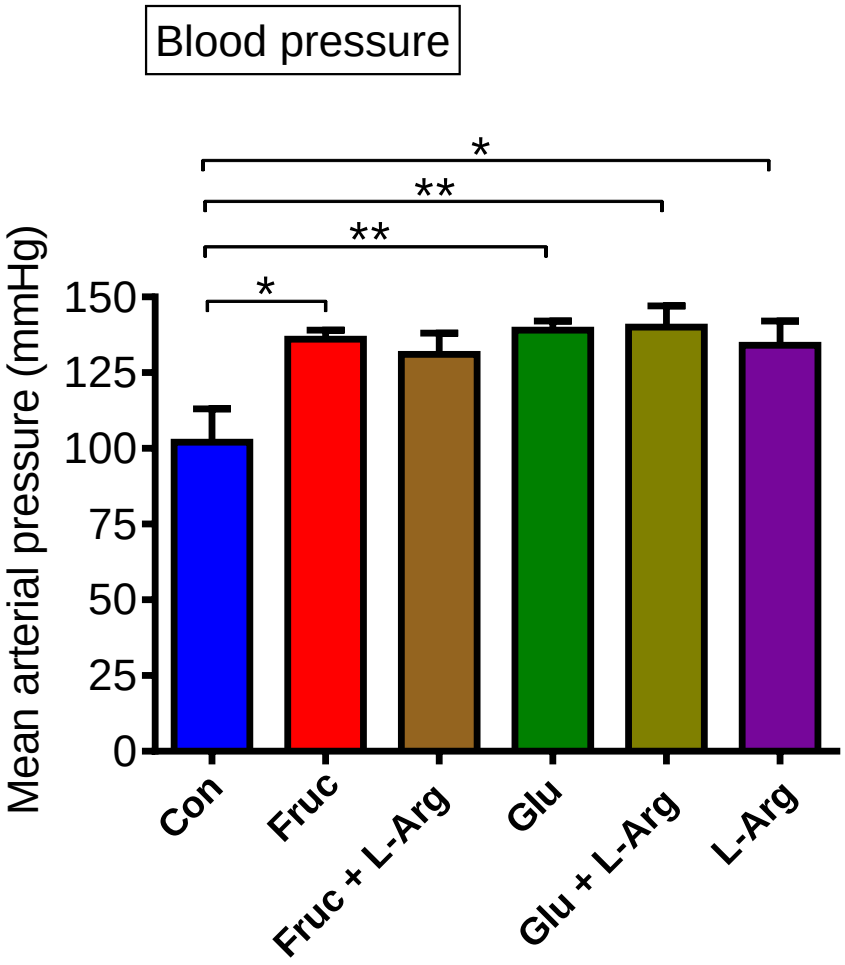

Figure 14. High glucose and high fructose diets increase the mean arterial pressure in rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg, 500 mg/kg body weight in drinking water) for 12 weeks. Mean arterial pressure was measured from the carotid artery in the anesthetized rats. *P<0.05, **P < 0.01. (n = 6-8).

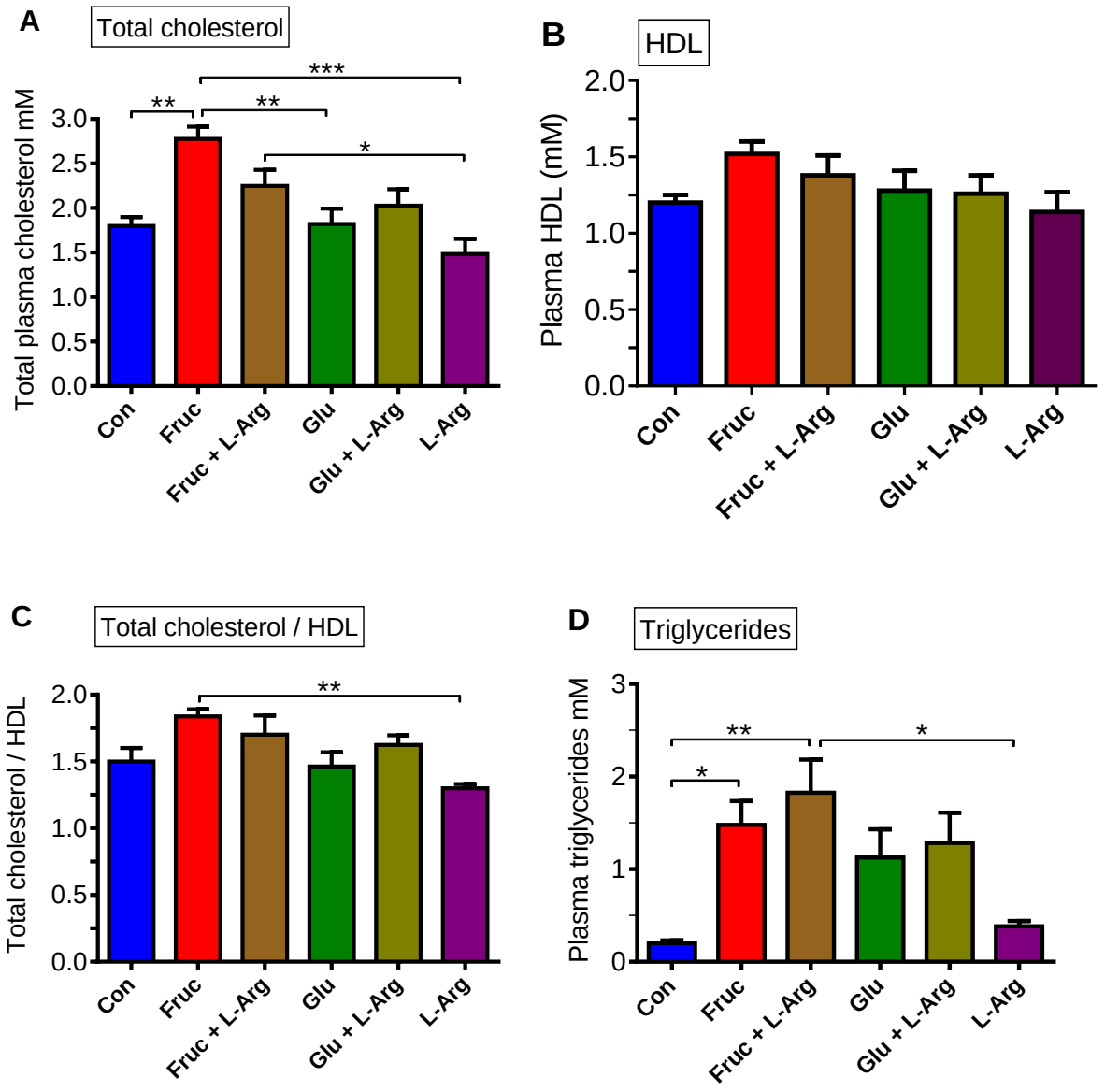

Figure 15. A high fructose diet increases plasma total cholesterol and triglyceride levels in rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. Plasma total cholesterol, HDL and triglycerides were measured by the Clinical Chemistry Lab. **P*<0.05, ***P*<0.01, ****P*<0.001. (*n* = 6-8).

High glucose and high fructose diets significantly increase plasma methylglyoxal levels

Treatment of rats for 12 weeks with either a high glucose or a high fructose diet significantly increased the plasma levels of MG compared to the control group (Fig. 16). Co-treatment with L-arginine attenuated glucose-induced, but not fructose-induced increase in plasma MG levels. Surprisingly, treatment with L-arginine alone also caused a significant increase in plasma MG compared to the control group (Fig. 16).

High glucose and high fructose diets significantly increase methylglyoxal levels in the aorta and mesenteric vascular bed in rats

Treatment of rats for 12 weeks with either a high glucose or a high fructose diet significantly increased the MG levels in the aorta (Fig. 17) and the mesenteric vascular bed (Fig. 18) compared to the control group. Co-treatment with L-arginine attenuated glucose-induced as well as fructose-induced increase in the MG levels in both tissues. Treatment with L-arginine alone did not increase in the MG levels in these vessels (Figs. 17, 18).

High glucose and high fructose diets did not affect methylglyoxal or glutathione levels in the brain, the heart and the kidneys in rats

Treatment of rats for 12 weeks with either a high glucose or a high fructose diet, either alone or combined with L-arginine, did not affect the MG levels or the GSH levels in the brain (Fig. 19), the heart (Fig. 20) or the kidneys (Fig. 21), compared to the control group. Treatment with L-arginine alone also did not affect the MG levels in these three organs.

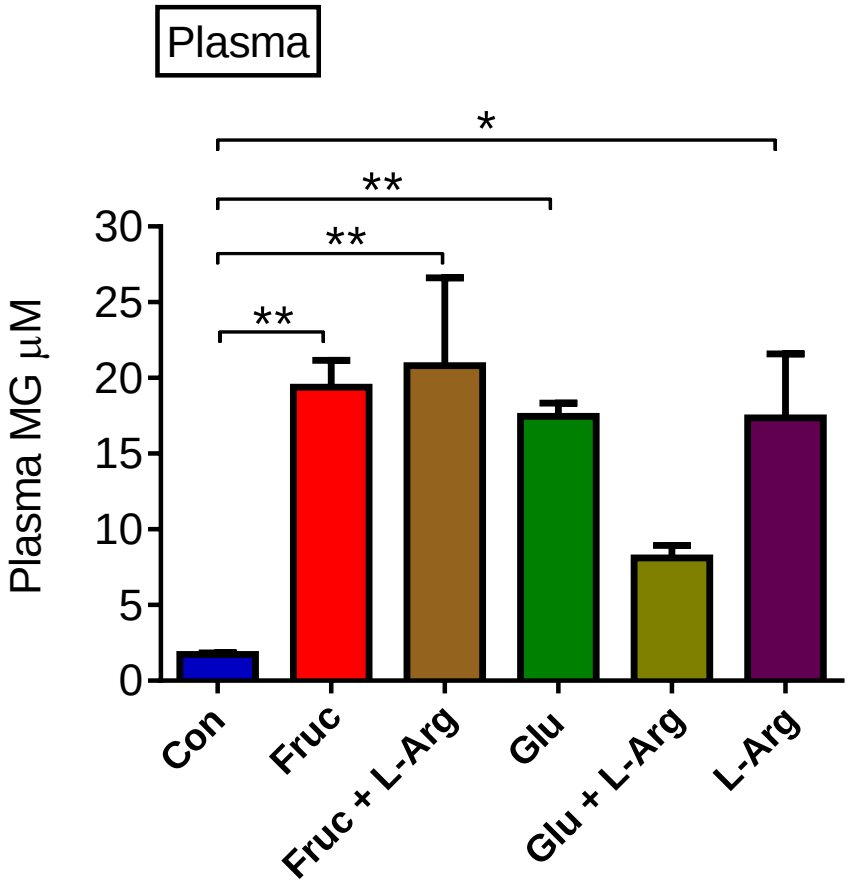

Figure 16. High fructose and high glucose diets increase plasma methylglyoxal (MG) in rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. Plasma MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. **P*<0.05, ***P*<0.01. (*n* = 6-8).

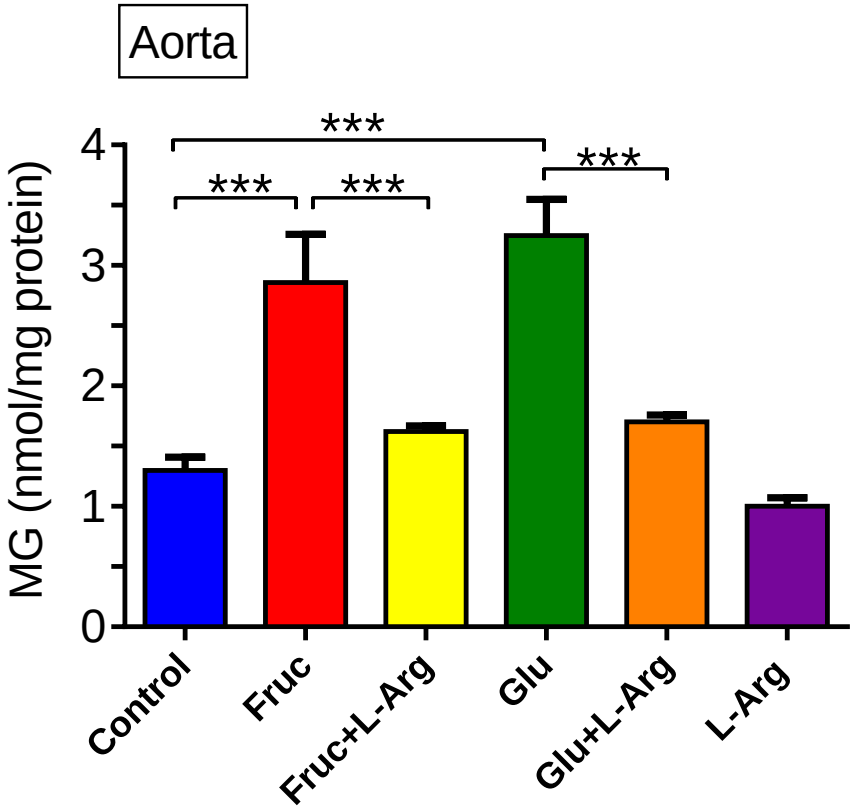

Figure 17. High fructose and high glucose diets increase methylglyoxal (MG) in the aorta of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The aorta was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. ***P<0.001. (n = 6-8).

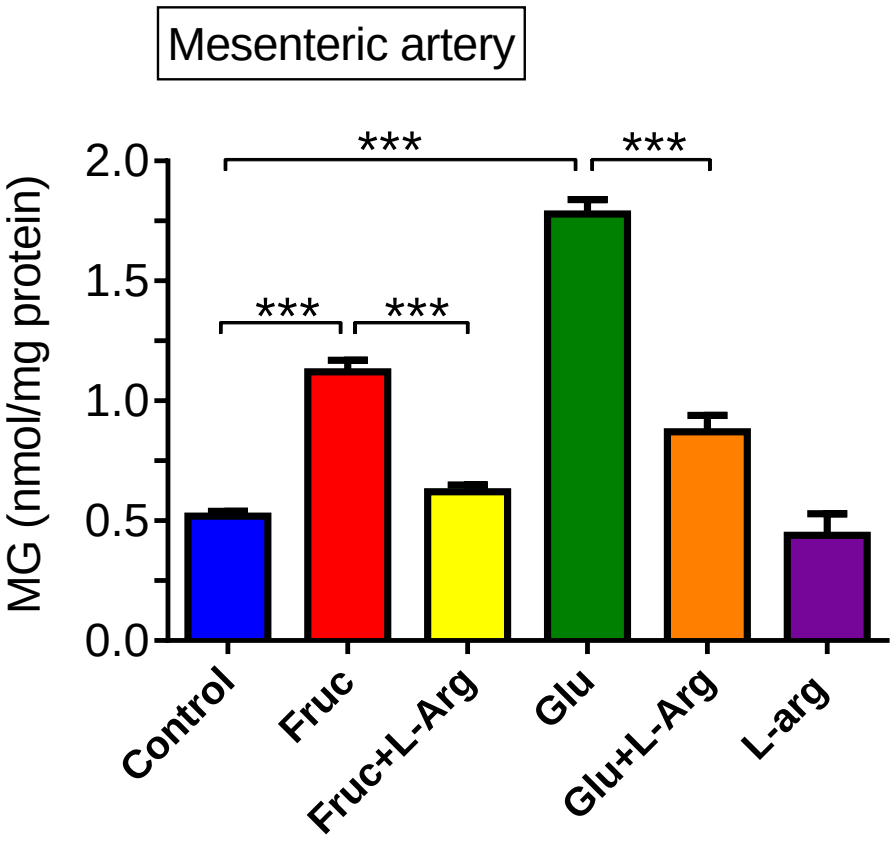

Figure 18. High fructose and high glucose diets increase methylglyoxal (MG) in the mesenteric artery of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The mesenteric vessels were homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. ****P*<0.001. (*n* = 6-8).

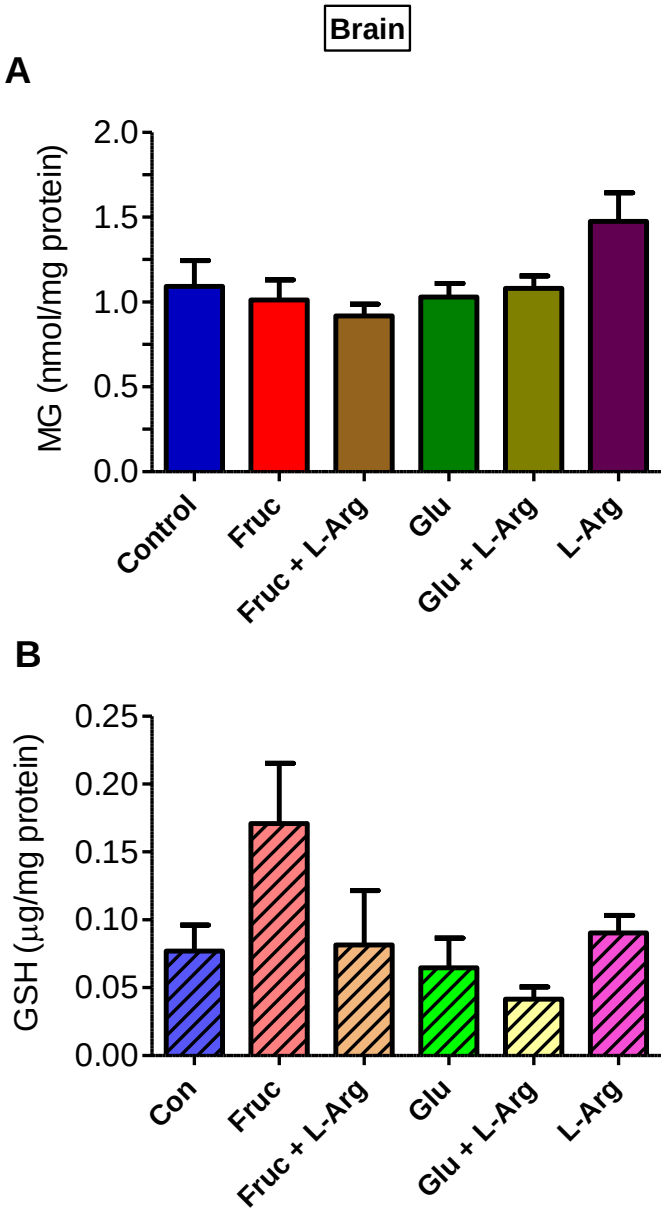

Figure 19. High fructose and high glucose diets do not affect methylglyoxal (MG) or reduced glutathione (GSH) levels in the brain of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The brain was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. (n = 6-8).

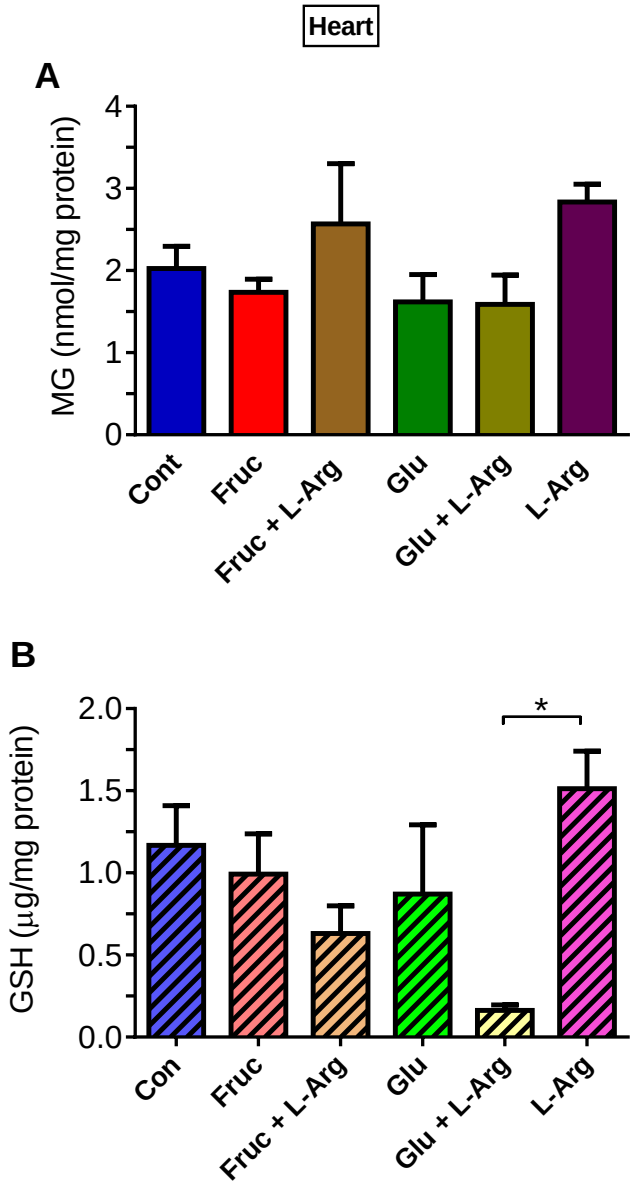

Figure 20. High fructose and high glucose diets do not affect methylglyoxal (MG) or reduced glutathione (GSH) levels in the heart of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The heart was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. *P<0.05. (n = 6-8).

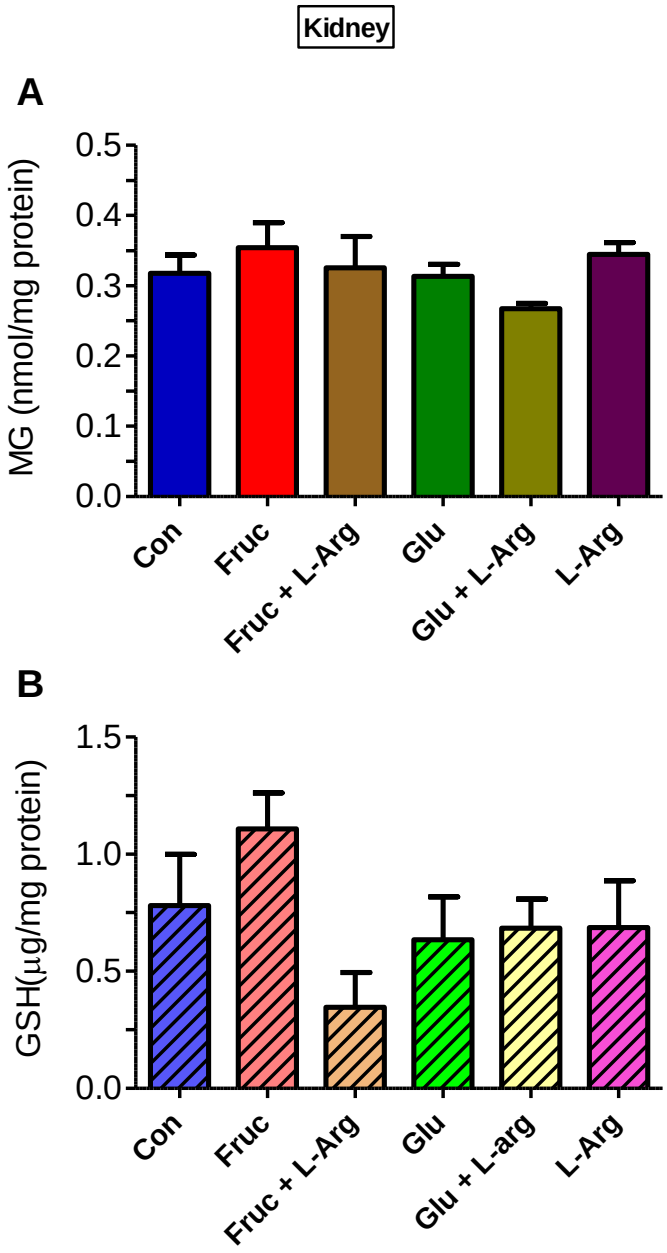

Figure 21. High fructose and high glucose diets do not affect methylglyoxal (MG) or reduced glutathione (GSH) levels in the kidney of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The kidney was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. (*n* = 6-8).

A high fructose diet, but not a high glucose diet, significantly increases methylglyoxal and glutathione levels in the liver in rats

Treatment of rats for 12 weeks with either a high fructose diet significantly increased the MG levels and the GSH levels in the liver compared to the control and the glucose treated groups (Fig. 22). Co-treatment with L-arginine attenuated the fructose-induced increase in GSH levels but not MG levels. Treatment with a high glucose diet, alone or combined with L-arginine, or treatment with L-arginine alone did not affect the MG and GSH levels in the liver, compared to the control group (Fig. 22).

L-arginine significantly increases methylglyoxal levels and decreases glutathione levels in the skeletal muscles in rats

Treatment of rats for 12 weeks with L-arginine alone (500 mg/kg body wt in drinking water) significantly increased the MG levels and decreased the GSH levels in the skeletal muscle, compared to the control group (Fig. 23). Treatment with a high fructose diet combined with L-arginine also significantly increased the MG level, but did not affect the GSH level, compared to the control group. A high glucose diet, alone or combined with L-arginine, did not affect the MG or GSH levels in the skeletal muscle (Fig. 23).

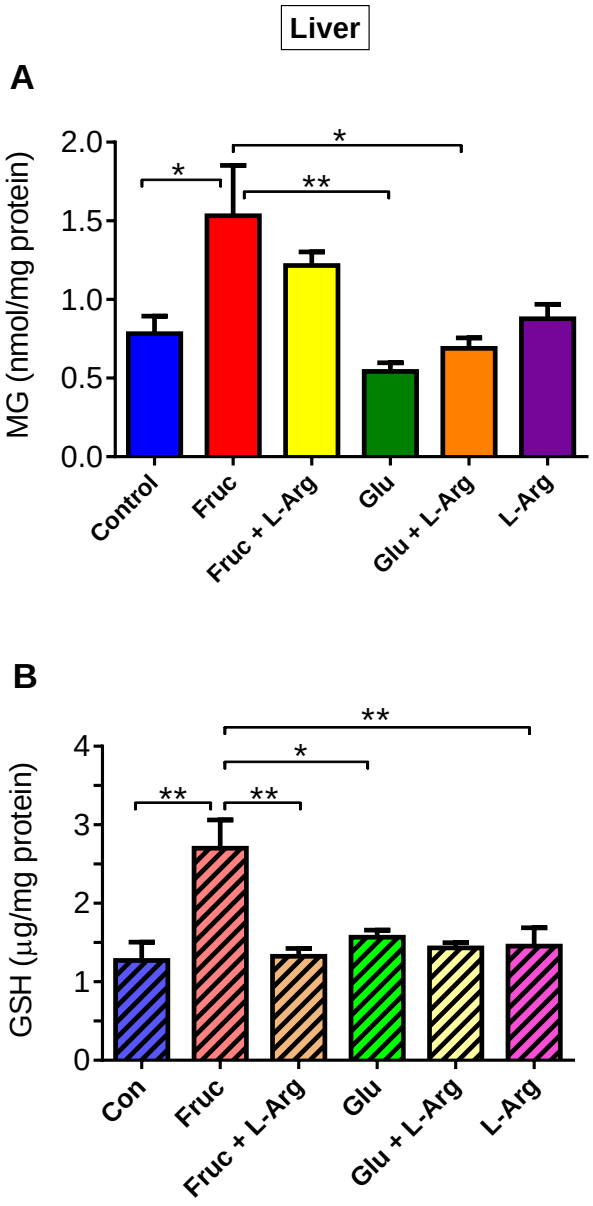

Figure 22. A high fructose diet, but not a high glucose diet, increases methylglyoxal (MG) and reduced glutathione (GSH) levels in the liver of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The liver was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. *P<0.05, **P<0.01. (n = 6-8).

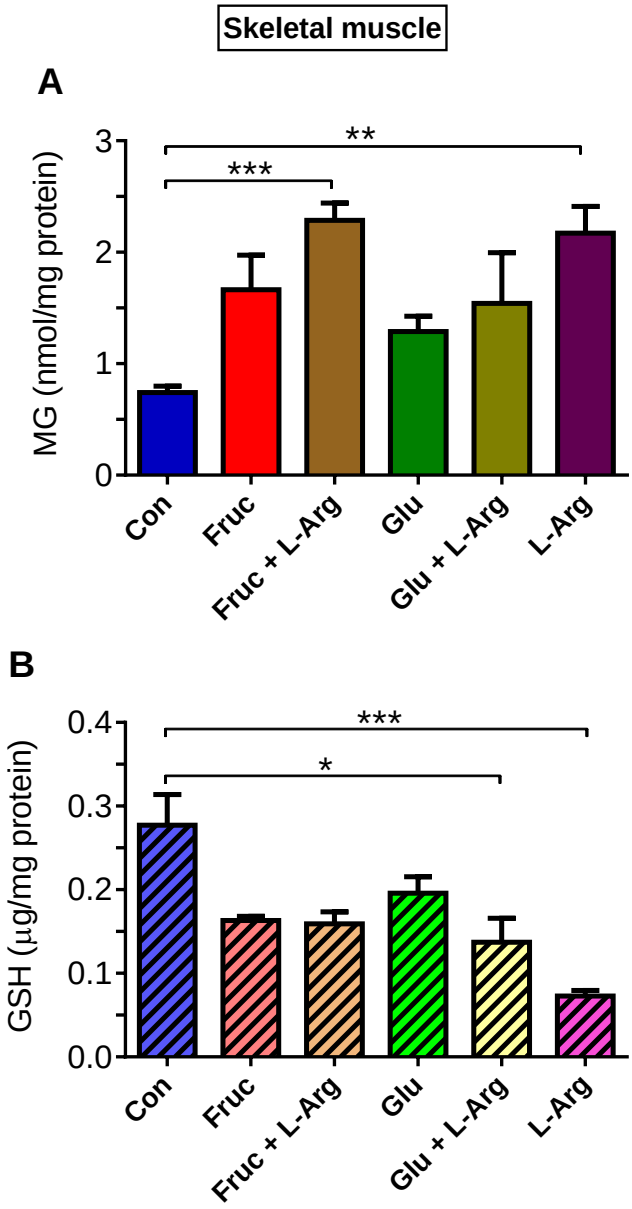

Figure 23. L-arginine increases methylglyoxal (MG) and decreases reduced glutathione (GSH) levels in the skeletal muscle of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The skeletal muscles were homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. *P<0.05, **P<0.01, ***P<0.001. (n = 6-8).

A high fructose diet, but not a high glucose diet, significantly decreases methylglyoxal and increases glutathione levels in the adipose tissue in rats

Treatment of rats for 12 weeks with a high fructose diet significantly decreased the MG levels and increased the GSH levels in the adipose tissue, compared to the control group (Fig. 24). Co-treatment with L-arginine attenuated the fructose-induced decrease in MG level and increase in GSH level. Treatment with a high glucose diet, alone or combined with L-arginine, or treatment with L-arginine alone did not affect the MG and GSH levels in the adipose tissue, compared to the control group (Fig. 24).

A high fructose diet significantly decreases methylglyoxal levels, whereas a high glucose diet increases glutathione levels, in the small intestine in rats

Treatment of rats for 12 weeks with a high fructose diet significantly decreased the MG levels in the small intestine, without affecting the GSH levels, compared to the control group (Fig. 25). Co-treatment with L-arginine attenuated the fructose-induced decrease in MG levels. On the other hand, a high glucose diet significantly increased the GSH levels in the small intestine, without affecting the MG levels, compared to the control. Co-treatment with L-arginine attenuated the glucose-induced increase in GSH levels. Treatment with L-arginine alone did not affect the MG and GSH levels in the small intestine, compared to the control group (Fig. 25).

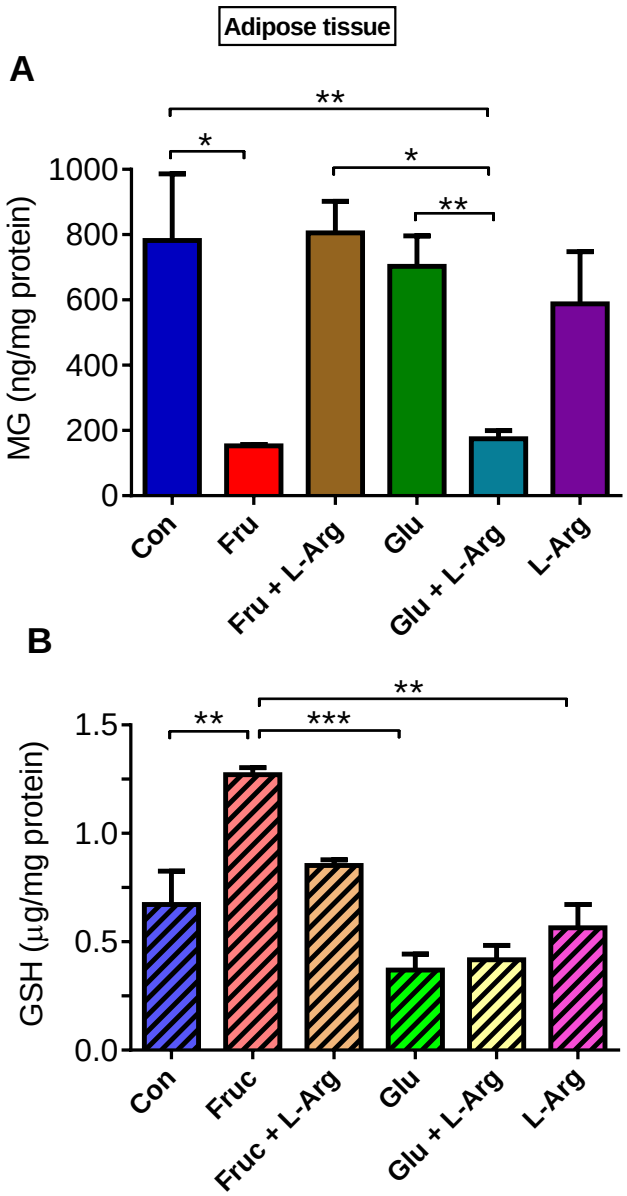

Figure 24. A high fructose diet, but not a high glucose diet, decreases methylglyoxal (MG) and increases glutathione (GSH) levels in the adipose tissue of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The adipose tissue was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. **P*<0.05, ***P*<0.01, ****P*<0.001. (*n* = 6-8).

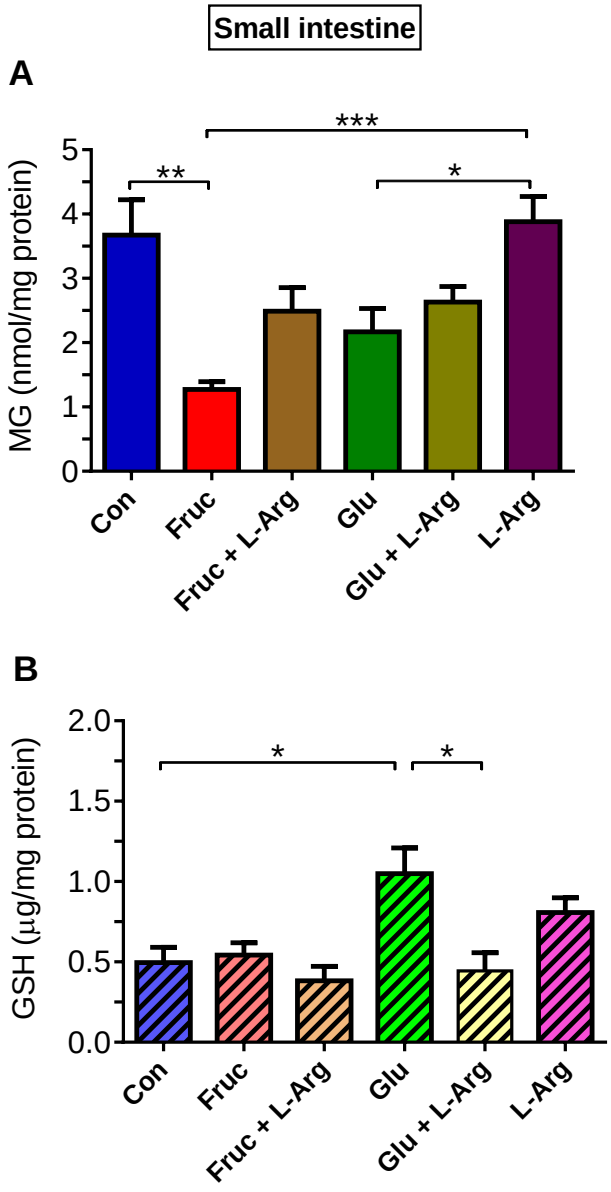

Figure 25. A high fructose diet decreases methylglyoxal (MG) level whereas a high glucose diet increases reduced glutathione (GSH) levels in the small intestine of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The small intestine was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. *P<0.05, **P<0.01, ***P<0.001. (n = 6-8).

A high fructose diet significantly increases methylglyoxal levels, compared to a high glucose diet, in the lungs in rats

Treatment of rats for 12 weeks with a high fructose diet significantly increased the MG levels in the lungs, compared to the high glucose diet treated and L-arginine treated groups, but not compared to the control group (Fig. 26). High fructose diet did not affect the GSH levels in the lungs. Co-treatment with L-arginine attenuated the fructose-induced increase in MG levels. Treatment with a high glucose diet, alone or combined with L-arginine, or treatment with L-arginine alone did not affect the MG and GSH levels in the lungs, compared to the control group (Fig. 26).

A high fructose diet significantly increases methylglyoxal levels, compared to a high glucose diet, in the spleen in rats

Treatment of rats for 12 weeks with a high fructose diet significantly increased the MG levels in the spleen, compared to the high glucose diet treated group but not compared to control (Fig. 27). The high fructose diet did not affect the GSH levels in the spleen. Co-treatment with L-arginine attenuated the fructose-induced increase in MG levels. Treatment with a high glucose diet, alone or combined with L-arginine, or treatment with L-arginine alone did not affect the MG and GSH levels in the spleen, compared to the control group (Fig. 27).

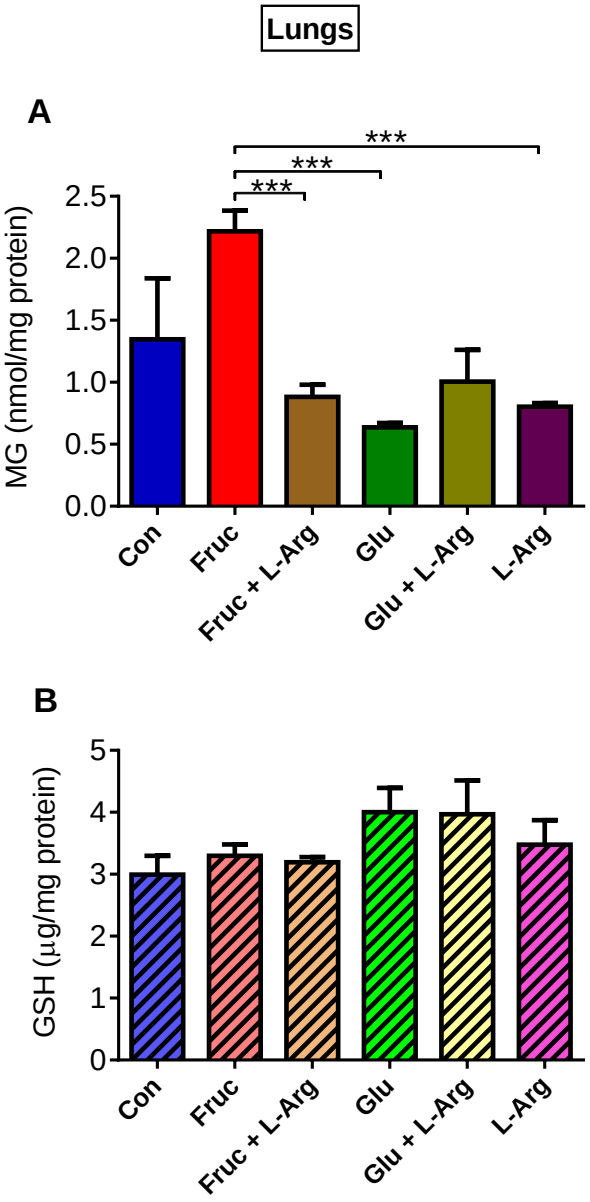

Figure 26. A high fructose diet increases methylglyoxal (MG) level, compared to a high glucose diet, in the lungs of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The lungs were homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. ***P<0.001. (n = 6-8).

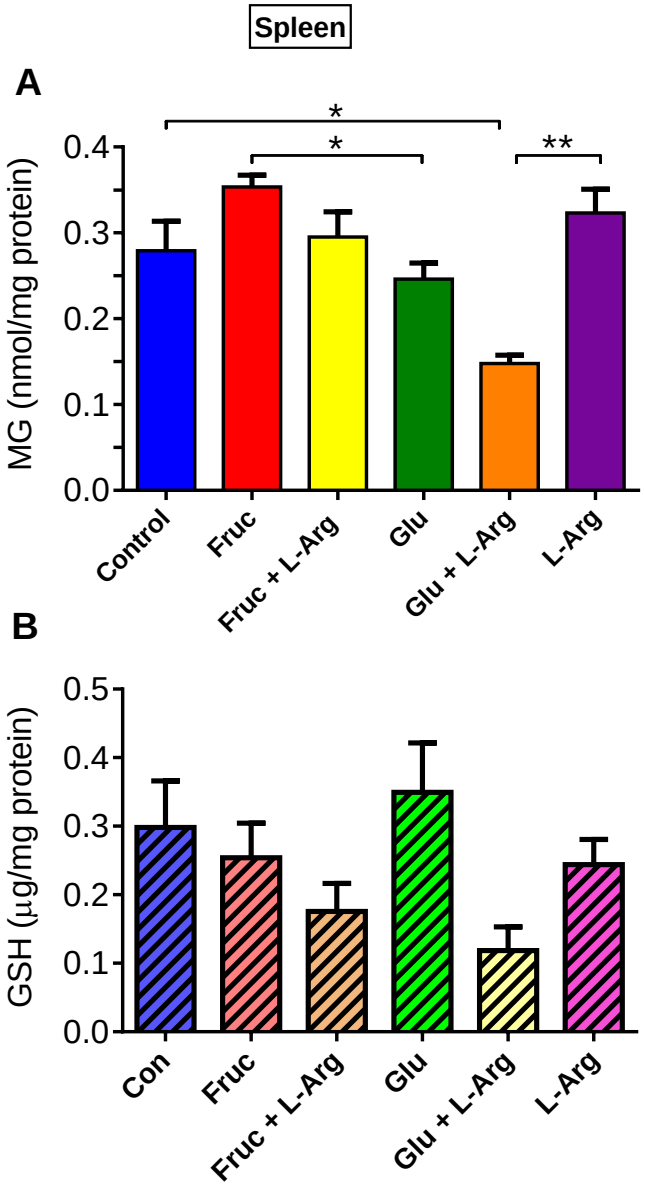

Figure 27. A high glucose diet decreases methylglyoxal (MG) level, compared to a high fructose diet, in the spleen of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The spleen was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. *P<0.05, **P<0.01. (n = 6-8).

A high glucose diet and L-arginine significantly increase methylglyoxal levels, compared to a high fructose diet, in the pancreas in rats

Treatment of rats for 12 weeks with a high glucose diet significantly increased the MG levels, compared to a high fructose diet, but not compared to the control group, in the pancreas (Fig. 28). Treatment with L-arginine alone also significantly increased the MG levels in the pancreas, compared to the control and high fructose groups. Treatment with a high fructose diet did not affect the MG levels, compared to the control group (Fig. 28). GSH levels were not measured in the pancreas.

High glucose and high fructose diets did not affect methylglyoxal levels in the urine in rats

Treatment of rats for 12 weeks with either a high glucose or a high fructose diet, either alone or combined with L-arginine, did not affect the MG levels in the urine (Fig. 29), compared to the control group. The GSH levels in the urine were not measured.

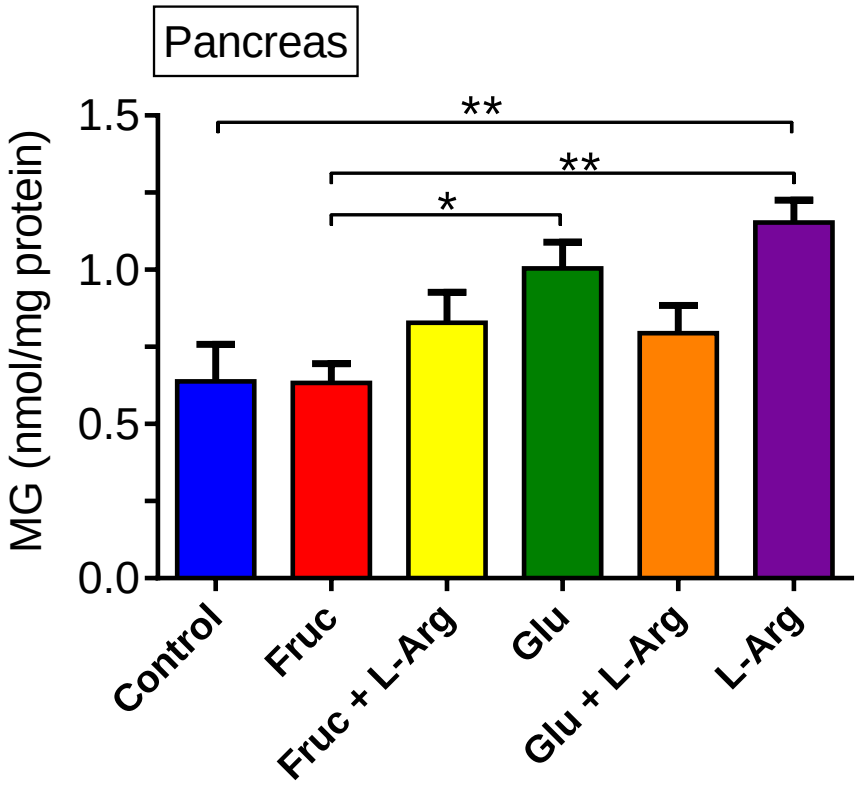

Figure 28. A high glucose diet and L-arginine increase methylglyoxal (MG) levels, compared to a high fructose diet, in the pancreas of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. The pancreas was homogenized and MG was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. *P<0.05, **P<0.01. (n = 6-8).

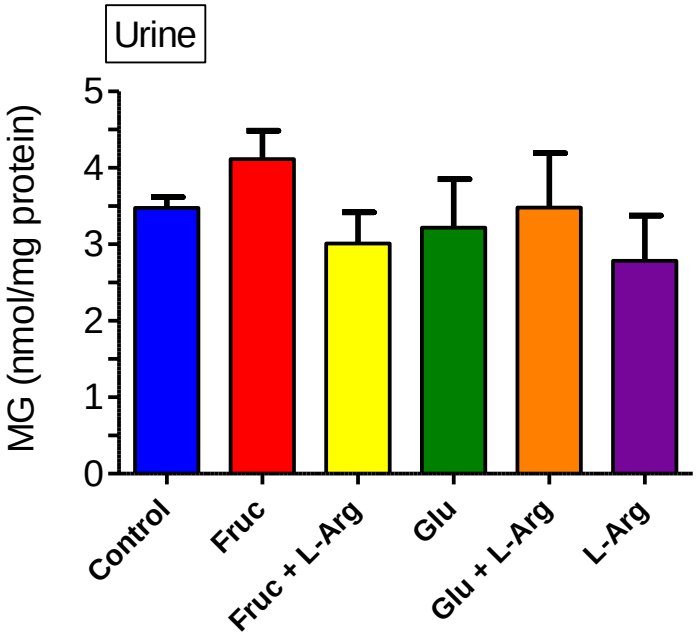

Figure 29. High fructose and high glucose diets do not affect methylglyoxal (MG) levels in the urine of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. At the end of 12 weeks the rats were fasted overnight and the urine was collected in individual metabolic cages. The MG in the urine was derivatized with o-phenylenediamine to form 2-methylquinoxaline which was measured by HPLC. (n = 6-8).

L-arginine significantly decreases fasting plasma insulin levels in rats

Treatment of rats for 12 weeks with L-arginine alone (500 mg/kg body wt in drinking water) significantly decreased the fasting plasma insulin levels, compared to the control group (Fig. 30). Treatment with a high fructose diet or a high glucose diet, alone or combined with L-arginine, had no effect on fasting plasma insulin or glucose levels (Fig. 30).

A high glucose and high fructose diets do not affect plasma glucose and insulin area under curves (AUCs) following an oral glucose tolerance test in rats

Treatment of rats for 12 weeks with either a high fructose or a high glucose diet, alone or combined with L-arginine, did not affect the oral glucose tolerance test (Fig. 31). However, treatment with L-arginine alone (500 mg/kg body wt in drinking water) significantly decreased the insulin AUC, compared to the control group (Fig. 31, 32). Following an oral glucose load (1 g/kg body wt), plasma glucose and insulin levels were determined at specific times for upto 2 h. The AUCs were determined for the glucose and insulin curves.

Despite this, treatment with a high glucose diet significantly decreased plasma insulin levels at 15 and 30 min following the glucose load, compared to the control group at respective times, without affecting the plasma glucose levels (Fig. 33). Moreover, co-treatment with high glucose and L-arginine, significantly decreased plasma insulin at 30 and 120 min, compared to the control group at respective times (Fig. 33).

Treatment with a high fructose diet significantly decreased plasma insulin levels at 30 min following the glucose load, compared to the control group at respective time, without affecting the plasma glucose levels (Fig. 34).

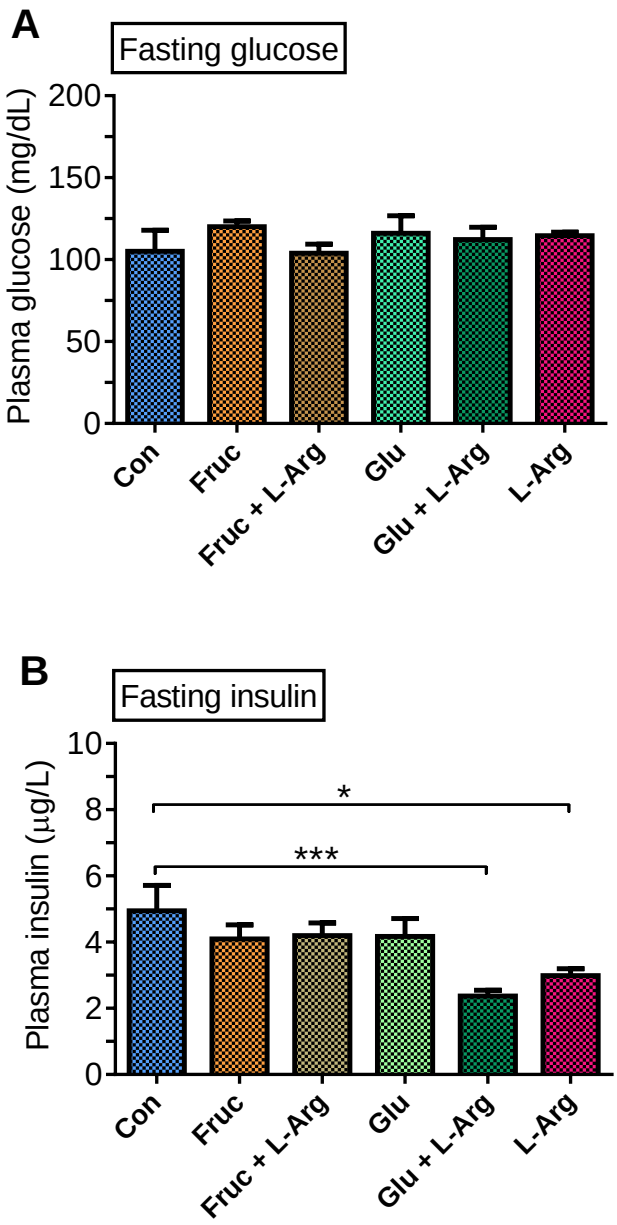

Figure 30. L-arginine decreases fasting plasma insulin levels of rats. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high fructose (Fruc, 60%) diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. At the end of 12 weeks the rats were fasted overnight and fasting blood sample was collected. Plasma glucose and insulin were measured with assay kits. *P<0.05, ***P<0.001. (n = 6-8).

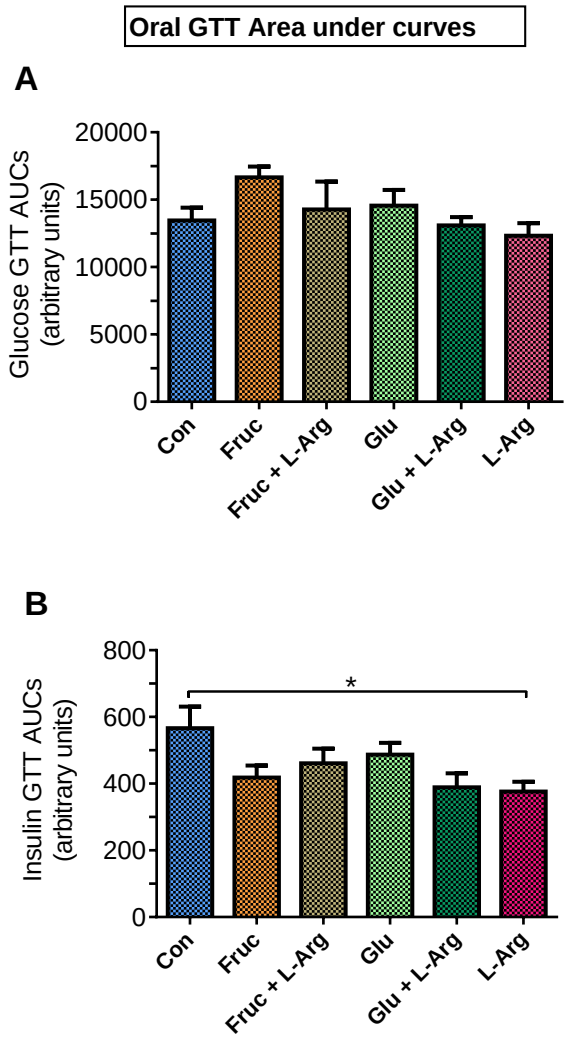

Figure 31. L-arginine reduces plasma insulin response following an oral glucose tolerance test. Male 10 week old Sprague-Dawley rats were treated with either a control diet, a high glucose (Glu, 60%) or a high fructose (Fru, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. At the end of 12 weeks the rat was fasted overnight and fasting blood sample was collected from anesthetized rat. An oral glucose solution was administered at 1 g/kg body wt and further blood samples were collected from a carotid artery cannula at specific times. Plasma glucose and insulin were measured with assay kits. The area under curves (AUCs) were determined for the glucose and insulin plasma levels determined at specific times for up to 2 h. *P<0.05. (n = 6-8).

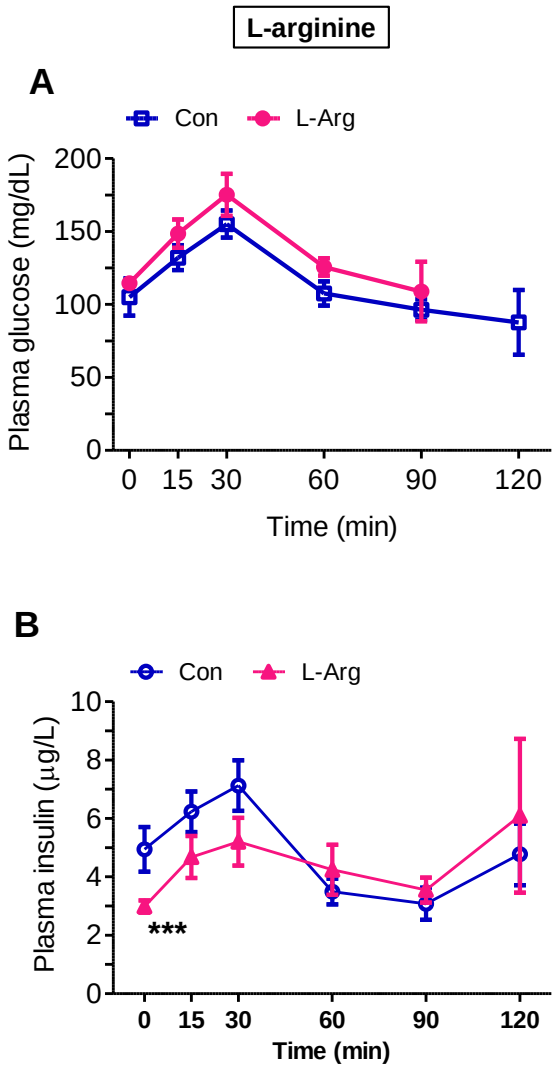

Figure 32. L-arginine does not affect glucose tolerance following an oral glucose tolerance test. Male 10 week old Sprague-Dawley rats were treated with either a control diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. At the end of 12 weeks the rat was fasted overnight and fasting blood sample was collected from anesthetized rat. An oral glucose solution was administered at 1 g/kg body wt and further blood samples were collected from a carotid artery cannula at specific times. Plasma glucose and insulin were measured with assay kits. ***P<0.01 *vs.* control at respective time point. (n = 6-8).

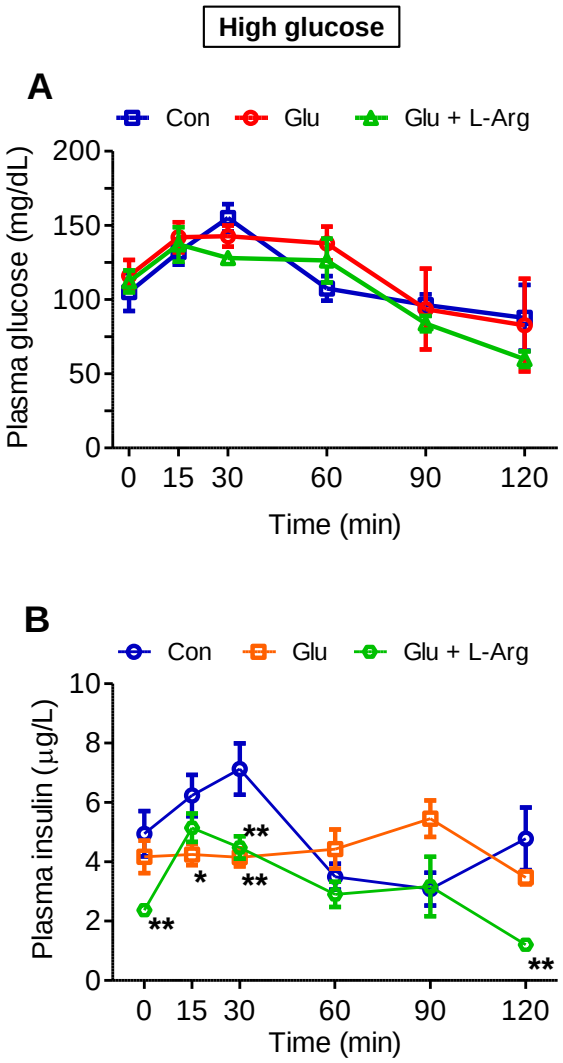

Figure 33. A high glucose diet alone or combined with L-arginine decrease plasma insulin response levels to an oral glucose tolerance test. Male 10 week old Sprague-Dawley rats were treated with either a control diet or a high glucose (Glu, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. At the end of 12 weeks the rat was fasted overnight and fasting blood sample was collected from anesthetized rat. An oral glucose solution was administered at 1 g/kg body wt and further blood samples were collected from a carotid artery cannula at specific times. Plasma glucose and insulin were measured with assay kits. *P<0.05, **P<0.01 *vs.* control at respective time point. (n = 6-8).

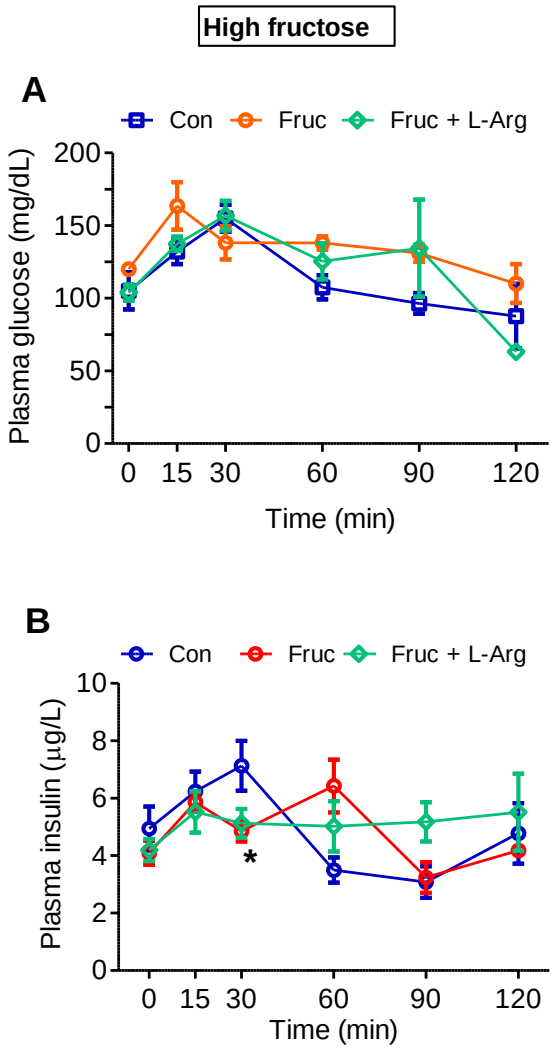

Figure 34. A high fructose diet alone or combined with L-arginine do not affect glucose tolerance following an oral glucose tolerance test. Male 10 week old Sprague-Dawley rats were treated with either a control diet or a high fructose (Fru, 60%) diet alone or combined with the methylglyoxal scavenger, L-arginine (L-Arg,500 mg/kg body wt) for 12 weeks. At the end of 12 weeks the rat was fasted overnight and fasting blood sample was collected from anesthetized rat. An oral glucose solution was administered at 1 g/kg body wt and further blood samples were collected from a carotid artery cannula at specific times. Plasma glucose and insulin were measured with assay kits. *P<0.05 *vs*. control at respective time point. (n = 6-8).

Parameter	Fruc	Fruc + LA	Gluc	Gluc + LA	LA
Bodyweight	↔	↔	↔	↔	↔
BP	↑	↑	↑	↑	↑
TC	↑ vs. C, G	↑ vs. LA	↔	↔	↔
HDL	↔	↔	↔	↔	↔
TC/HDL	↔	↔	↔	↔	↔
Triglycerides	↑	↑ vs. C, LA	↔	↔	↔
← **MG levels** →					
Plasma	↑	↑	↑	↔	↑
Aorta	↑ vs. C, FA	↔	↑ vs. C, GA	↔	↔
Mesent. artery	↑ vs. C, FA	↔	↑ vs. C, GA	↔	↔
Brain	↔	↔	↔	↔	↔
Heart	↔	↔	↔	↔, ↓ vs. LA	↔
Kidney	↔	↔	↔	↔	↔
Liver	↑ vs. C, G	↔	↔, ↓ vs. F	↔	↔
Skeletal muscle	↔	↑	↔	↔	↑
Adipose tissue	↓	↔	↔, ↑ vs. GA	↔	↔
Small intestine	↓	↔	↔	↔	↔, ↑ vs. G
Lungs	↑ vs. FA	↔	↔	↔	↔
Spleen	↑ vs. G	↔	↔	↓ vs. C, LA	↔
Pancreas	↔	↔	↑ vs. F	↔	↑ vs. C, F
Urine	↔	↔	↔	↔	↔
← **GSH levels** →					
Brain	↔	↔	↔	↔	↔
Heart	↔	↔	↔	↓ vs. LA	↔
Kidney	↔	↔	↔	↔	↔
Liver	↑ vs. C, FA, G, LA	↔	↔	↔	↔
Skeletal muscle	↔	↔	↔	↓	↓
Adipose tissue	↑ vs. C, G, LA	↔	↔	↔	↔
Small intestine	↔	↔	↑ vs. C, GA	↔	↔
Lungs	↔	↔	↔	↔	↔
Spleen	↔	↔	↔	↔	↔
Fasting levels and OGTT test					
Fasting glucose	↔	↔	↔	↔	↔
Fasting insulin	↔	↔	↔	↓	↓
OGTT insulin	↔	↔	↔	↔	↓

Table 3. Summary of results

(see next page)

Abbreviations: BP - blood pressure; C – control; F, Fruc - high fructose; G, Gluc - high glucose; GSH - reduced glutathione; HDL - high density lipoprotein; LA - L-arginine; MG – methylglyoxal; OGTT - oral glucose tolerance test; TC – triglycerides.

Symbols: ↔ no change compared to control; ↑ increase or ↓ decrease compared to control unless otherwise stated; < less than; > more than.

Discussion

MG is an important metabolite produce, probably almost continuously, in a variety of cells, organisms and humans, when they metabolize glucose. Research on MG has been going on for almost the last hundred years. Yet, some fundamental knowledge about MG is lacking. For example, to the best of our knowledge there is no report of intracellular levels of MG in almost any cell type. One probable reason for this lack of knowledge could be that MG is reactive and as soon as it is produced it most likely reacts with nearby molecules which have an affinity for it as a result of an exposed reactive group. Thus, it is doubtful if small amounts of MG generated inside a cell ever cross the cell membrane and leave the cell. The degradation of small amounts of MG is efficiently carried out mainly by the glyoxalase enzymes with the help of GSH (Thornalley, 1998; Vander Jagt & Hunsaker, 2003). The cysteine in GSH binds to MG and presents it to glyoxalase 1 as discussed in the introduction. Thus, the cellular levels of GSH are intricately related to the levels of MG. Adequate amounts of GSH are likely keeping the MG levels to a minimum and prevent its harmful reactions under normal physiological conditions. It also not known whether or how much MG is transported across cell membranes or whether it is absorbed after oral administration. One study had reported administration of MG in drinking water for 18 weeks, to study its effect on blood pressure (Vasdev et al, 1998; Vasdev & Stuckless, 2010). It was administered in a concentration of 0.2% from 0 to 5 weeks, 0.4% from 6 to 10 weeks and 0.8% from 11 to 18 weeks. However, the plasma or aortic or renal MG levels were not measured, which might have given a clue about the oral absorption of MG. MG also reacts with epithelial cells of the intestines and the colon (Baskaran & Balasubramanian, 1990) which makes its oral bioavailability questionable. Regarding transport across cell membranes one study has shown that incubation of cultured L6 muscle cells resulted in only 3.1 ± 0.39% of

the 2.5 mM incubated MG to enter the cell (Riboulet-Chavey et al, 2006). Of this about 63% was in the membrane fraction and about 33% in the cytosolic fraction. Similarly, another study showed that incubation of cultured rat aortic smooth muscle cells with 160 μM MG resulted in only 1.8% being incorporated in the cells (Che et al, 1997). These results show that very little MG crosses membranes and when it does most of it binds to the membrane, which can be explained by its reactivity.

MG has been shown to disrupt the cycle of GSH:GSSG and also reduces the activity and expression of enzymes involved in glutathione homeostasis, such as glutathione reductase (Blakytny & Harding, 1992) and glutathione peroxidase (Paget et al, 1998). This would reduce the levels of GSH (Kikuchi et al, 1999; Shinpo et al, 2000; Wu & Juurlink, 2002), which in turn would reduce the degradation of MG and set up a vicious cycle. Thus, it is logical to measure GSH alongwith MG in any determinations. Levels of one would most likely affect the other in long-term regulation.

Our lab had measured and reported the basal levels of MG in several different organs/tissues in 12 week old male Sprague-Dawley rats (Dhar et al, 2010a). One surprising finding was the apparent high levels of basal MG in the aorta, compared to other organs such as the heart, brain, liver, kidney and the lungs. The lab examined the absorption and distribution of MG to different organs after intraperitoneal administration of 17.25 mg/kg body wt. This study provided important clues about the transport of MG in and out of the blood. There was a further significant increase in the aortic MG levels over the basal level, which was attenuated by the MG scavenger alagebrium. There was also a significant increase in the MG levels in the heart, kidney, liver and the lungs over the basal levels, but not in the spleen and the brain. No increase in the MG levels in the brain can be explained by the difficulty of blood MG to cross the blood brain barrier. The

urinary MG levels increased significantly after i.p. administration indicating excretion of blood MG into the urine. This would suggest that excess MG in the blood may be filtered rapidly by the glomerulus before it reacts with molecules in the blood, although this is just a conjecture.

The results of my study proved my first hypothesis that high fructose and high glucose diets produce different amounts of MG in some organs such as the liver, adipose tissue, small intestine, lungs, pancreas and the spleen. The difference was markedly significant in the liver and the pancreas. In some organs and the plasma it was not significantly different between high fructose and high glucose diets. Thus, the metabolism of these two monosaccharides in different organs, in terms of MG production requires further studies.

The results did not support my second hypothesis that the GSH levels in different organs/tissues will be reciprocal to the MG levels. However, in a couple of organs a reciprocal relationship between MG and GSH levels was observed. For example, MG decreased whereas GSH increased in the adipose tissue in high fructose treated rats (Fig. 24). Also, MG increased in the skeletal muscle whereas GSH decreased in L-arginine treated rats (Fig. 23). Other factors in the relationship between MG and GSH need to be investigated. For example, the level of antioxidant defenses and oxidative stress could affect GSH levels and GSH response to any increase in MG production.

The results did not reveal any impaired glucose tolerance or insulin resistance in high glucose and high fructose treated rats, so any relationship between MG levels in the pancreas, adipose tissue, skeletal muscle and plasma, and impaired glucose tolerance could not be established conclusively.

Regarding my fourth hypothesis that L-arginine will attenuate the increase in MG levels by acting as a scavenger, I obtained mixed results. Thus, L-arginine attenuated the increase in MG

levels caused by high fructose or high glucose diets in the plasma (in case of high glucose diet), and in some organs/tissues such as the aorta, mesenteric vessels, liver and lungs (in case of high fructose). On the other hand the increase in MG levels caused by a high fructose diet in the plasma was not attenuated by L-arginine.

Discussing the results further, my study showed that both, high glucose and high fructose diets, significantly increased the blood pressure as expected and as reported previously in other studies (Hwang et al, 1987; Jalal et al, 2010; Kaufman et al, 1991; Madero et al, 2011a; Madero et al, 2011b; Wang et al, 2008). This increase in blood pressure parallels the significant increase in plasma MG levels caused by high fructose and high glucose diets (Fig. 16). The association of increased blood pressure with the significant increase in aortic and mesenteric vascular bed MG levels caused by high glucose and high fructose diets (Figs. 17 and 18) can also be considered in studies of cause and effect. One interesting and surprising finding was that the MG scavenger L-arginine did not attenuate the fructose- and glucose-induced increase in the blood pressure, and also that L-arginine itself caused a significant increase in the blood pressure. A surprising correlation is the fact that L-arginine also did not attenuate the increase in plasma MG levels caused by high fructose diet and L-arginine alone also caused a significant increase in plasma MG level (Fig. 16). It should be noted here L-arginine is a substrate for NO production (Palmer et al, 1988) in blood vessels and L-arginine supplements have been reported to lower the blood pressure (Dong et al, 2011). Thus, the increase in blood pressure and plasma MG levels by L-arginine is a puzzling finding and will be investigated further in our lab. It should also be pointed out that MG has been proposed as one of the causative factors in hypertension development in Sprague-Dawley rats by our lab, possibly by up regulating the renin angiotensin aldosterone system (Dhar et al, 2013; Dhar et al, 2014).

A high fructose diet, but not a high glucose diet, increased plasma total cholesterol and triglyceride levels (Fig. 15). These results can be viewed to have significance with the finding that a high fructose diet, but not a high glucose diet, significantly increases MG levels in the liver (Fig. 22), which is the main organ of cholesterol and triglyceride synthesis and release into the plasma in the form of very low density lipoproteins and high density lipoproteins. Co-treatment with high fructose and L-arginine prevented the increase in liver MG level and plasma total cholesterol level, but it did not attenuate the fructose-induced increase in triglyceride levels. L-arginine alone did not affect the plasma total cholesterol and triglyceride levels, which agrees with the finding that L-arginine alone had no effect on liver MG levels (Figs. 15 and 22). Our lab has shown that chronic treatment of Sprague-Dawley rats with MG for 4 weeks increased the expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and sterol regulatory element binding protein-2 (SREBP-2) in the liver (Huang, 2011), which can explain increased cholesterol synthesis and increased plasma total cholesterol level. HMG CoA reductase is the rate limiting enzyme in cholesterol synthesis in the liver. MG also increased the expression microsomal triglyceride transfer protein (MTP) (Huang, 2011), which plays a role in the synthesis of very low density lipoprotein (VLDL) in the liver. Increased VLDL synthesis can explain the increase in plasma triglyceride level.

High glucose and high fructose diets caused a significant increase in the aortic and mesenteric vascular bed MG levels (Figs. 17 and 18), which was attenuated by L-arginine. This increase in vascular MG can be linked with the fact that MG causes endothelial dysfunction in rat aorta and cultured rat aortic endothelial cells and human umbilical vein endothelial cells, which can be attenuated by two different MG scavengers, aminoguanidine and NAC, as shown by our lab (Dhar et al, 2010b). Moreover, endothelial dysfunction is a hallmark of diabetes (De

Vriese et al, 2000; Potenza et al, 2009). High glucose and hyperglycemia have also been shown to cause endothelial dysfunction (Du et al, 2001; Nishikawa et al, 2000; Potenza et al, 2009; Triggle, 2008). Putting all of these facts together, it is very likely that high glucose and high fructose diet induced increase in aortic and mesenteric vascular bed MG levels mediates endothelial dysfunction and hypertension development that is seen in diabetes. The mesenteric vascular bed is a resistance type vascular bed and is a part of blood pressure regulatory mechanisms. In this scenario, the attenuation of increase in MG levels by L-arginine in these vessels (Figs. 17 and 18) may possibly be benefited by increased NO production by L-arginine in preventing or attenuating high carbohydrate-diet-induced endothelial dysfunction. I did not measure endothelium-dependent relaxation or NO production in my study which can be considered a limitation of my study. Unfortunately, I could not measure GSH levels in the aorta and the mesenteric vascular bed because of a lack of enough tissue.

A high glucose or a high fructose diet did not affect the MG or GSH levels in the skeletal muscle (Fig. 23). The skeletal muscle is an insulin sensitive tissue for glucose uptake and utilization (Koistinen & Zierath, 2002). It is also a major site of insulin resistance that can contribute to hyperglycemia and development of type 2 diabetes mellitus (Koistinen & Zierath, 2002). Our lab has recently shown that acute or chronic treatment of Sprague-Dawley rats with MG causes insulin resistance or development of features of type 2 diabetes (Dhar et al, 2010a; Dhar et al, 2011). My results with the oral GTT did not show impaired glucose tolerance with either high glucose or high fructose diets (Figs. 30 to 34), which agrees with no increase in MG levels or decrease in GSH levels in the skeletal muscle due to a high glucose or high fructose diet. However, one surprising and puzzling finding was that L-arginine alone caused a significant increase in skeletal muscle MG level and a decrease in GSH level. It has been shown that the

action of insulin in regulating skeletal muscle glucose uptake and utilization is partly dependent on NO-NOS pathway (Baron et al, 1995; Bradley et al, 2013), and since L-arginine is a substrate for NOS it can increase NO production, enhance skeletal muscle vasodilation and insulin-mediated glucose uptake. It remains to be determined whether L-arginine would influence glucose uptake by the skeletal muscle and thus contribute to increased metabolism and increased MG formation. Further investigation of this interesting fact is required. The decrease in GSH level in the skeletal muscle (Fig. 23) can be expected to contribute to the increase in MG level or *vice versa*.

The adipose tissue is another major insulin sensitive tissue and a site of high carbohydrate diet-induced lipogenesis. A high fructose diet significantly reduced the MG level and increased the GSH level in the adipose tissue (Fig. 24). Excess fructose is known to be lipogenic, especially in the liver (Park et al, 1992; Samuel, 2011). The high fructose as well as high glucose treated rats did not show a significant increase in body weights compared to the control group. The significance of decreased MG in the adipose tissue is difficult to interpret in terms of lipogenesis. However, the increase in adipose tissue GSH level may be an adaptive compensatory response which prevented an increase in MG levels in this tissue. High glucose and L-arginine alone did not affect the adipose tissue MG and GSH levels.

A high fructose diet treated rats had significantly lower MG level whereas a high glucose diet treated rats had significantly higher GSH level, compared to the control group in the small intestine (Fig. 25). The higher GSH level in the high glucose treated rats may have attenuated an increase in MG levels in the small intestine. The functional or metabolic significance of low MG level in the high fructose diet treated rats is difficult to explain without further studies on the absorption and metabolism of these monosaccharides in the intestinal wall. The expression of

glucose and fructose transporters such as sodium-dependent glucose transporters (SGLTs) and glucose transporters (GLUTs), especially GLUT 2 and GLUT 5 (Scheepers et al, 2004), in the small intestine, and the effect of a high fructose and a high glucose diet and MG levels on these transporters needs to be studied.

A high fructose diet significantly increased the MG levels, compared to a high glucose diet, in the lungs and the spleen (Figs. 26 and 27). The GSH levels in these organs were not affected by the high fructose and high glucose diets. The functional significance of the increase in MG levels in these organs in not clear at present.

On the other hand a high glucose diet significantly increased the MG level in the pancreas, compared to a high fructose diet (Fig. 28). Our lab has recently shown the diabetogenic effects of chronic MG treatment in the pancreas of Sprague-Dawley rats (Dhar et al, 2011). L-arginine co-treatment attenuated the increase in pancreatic MG levels caused by high glucose. Surprisingly, L-arginine alone caused a significant increase in pancreatic MG level compared to the control group (Fig. 28). It is not clear if the effect of L-arginine is due to increased glucose uptake and metabolism by the pancreas. I could not measure pancreatic GSH level due to insufficient sample availability. The pancreas has been reported to have low antioxidant capability (Robertson & Harmon, 2007) and our lab has shown reduced GSH levels in the pancreas of chronic MG treated rats (Dhar et al, 2011).

High glucose and high fructose diets did not affect urinary MG levels (Fig. 29). It is possible that only a rapid increase in plasma MG levels increases urinary excretion of free MG as seen in the acute study with intraperitoneal administration of MG in our lab (Dhar et al, 2010a) (Fig. 4).

A high glucose and a high fructose diet for 12 weeks did not impair glucose tolerance as determined by the area under curves for the plasma glucose and insulin levels after an oral GTT

(Fig. 31), It is worth noting that high glucose diet treated rats had significantly lower plasma response at 15 and 30 min following an oral glucose load for the GTT (Fig. 33). This finding can be linked with the higher pancreatic MG level induced by high glucose. The lack of impairment of glucose tolerance in high glucose and high fructose treated rats can be viewed to agree with a lack of significant increase in MG levels in the skeletal muscle and adipose tissue, two major sites of insulin resistance, in these two treatment groups. Although chronic high fructose treatment has been reported to cause insulin resistance (Hwang et al, 1987; Wang et al, 2008), my results did not show any glucose intolerance.

L-arginine alone as well as in combination with a high glucose diet significantly lowered fasting plasma insulin (Figs. 30, 32 and 33). L-arginine alone also significantly decreased the plasma insulin response following an oral GTT (Figs. 30, 32 and 33). This can be explained with the significantly higher pancreatic MG level caused by L-arginine alone (Fig. 28). It is possible that increased pancreatic MG is causing reduced insulin secretion as reported by our lab recently (Dhar et al, 2011).

Conclusions

High fructose and high fructose affect the MG as well as GSH levels differently in different organs possibly due to differences in their metabolism. The liver, adipose tissue and the skeletal muscle are the major organs that metabolize and store or utilize excess carbohydrates in the diet. The liver processes 80% of orally absorbed fructose and about 20% of orally absorbed glucose. An excess of fructose in the diet has also been shown to be harmful for the liver. Our results show a significant increase in MG levels in the liver in high fructose treated rats, but not in high glucose treated rats. The results thus support increased processing of fructose in the liver.

Fructose-induced increase in the liver GSH levels might be a compensatory response. The liver has the highest levels of GSH in the body. Glucose and fructose did not increase MG levels in the adipose tissue which might indicate storage of these carbohydrates as lipids rather than being metabolized for energy. The decreased MG in the adipose tissue in the fructose group maybe due to increased GSH levels. Similarly, the increased GSH in the small intestinal wall caused by high glucose diet might be keeping the MG levels within normal limits. The significant increase in plasma MG with high glucose and high fructose diets can be linked with a significant increase in the blood pressure. The significant increase in the aortic and mesenteric artery MG levels by high carbohydrate diets supports our previously demonstrated role of MG in causing endothelial dysfunction and increased blood pressure. The increased plasma cholesterol and triglyceride levels caused by a high fructose diet support conversion of excess fructose into triglycerides and the effect of MG on cholesterol production in the liver (our unpublished results). Also, the MG scavenger L-arginine, was inconsistent in attenuating the effects of high fructose and high glucose diets on different parameters measured. The adequacy of the dose of oral L-arginine was not confirmed with measurement of the plasma levels of L-arginine. Moreover, the oral GTT results were not conclusive, but hinted at impaired glucose tolerance in the high glucose diet group. High fructose is also known to impair glucose tolerance so the GTT needs to be repeated in further studies with high carbohydrate diets, to confirm the obesrved effects. Further studies to examine organ/tissue MG levels with organ specific functions and pathologies are warranted.

Significance of the study

The significance of the study is partially evident. For example, the increase in aortic and mesenteric vascular bed MG levels strengthens our labs findings that MG induces endothelial

dysfunction and also that high carbohydrate diets, especially hyperglycemia and diabetes cause endothelial dysfunction. High fructose diet treated rats had significantly elevated MG levels and also increased plasma total cholesterol and triglyceride levels which indicate that the harmful effect of high fructose on the liver and its lipogenic effect are most likely mediated by excess production of MG. This knowledge can be used to give appropriate advice about the harmful health effects of high fructose diets and soft drinks, a topic of great interest currently. HFCS is widely used in processed fruits and as believed by scientists, may be largely responsible for the current epidemics of obesity and type 2 diabetes. The significance of my study can be further established with further studies on investigations of the pathological effects of high carbohydrate diets and excess MG on different organs.

Limitations of the study

My study would have greatly benefited from functional studies in different organs which would have enabled me to directly correlate any changes in MG and GSH levels produced by high glucose and fructose diets with their pathological effects in that organ. For example, determination of endothelium dependent relaxation in isolated aortic and mesenteric artery rings would have enabled me to link any endothelial dysfunction with elevated MG levels in these vessels which were observed in my results. Similarly determination of expression of enzymes involved in the hepatic synthesis of cholesterol and triglycerides and very low density lipoprotein assembly would have enabled me to differentiate the effects of high fructose versus high glucose diets on these enzymes and the role of MG in these effects. One of my aims was to see if L-arginine was effective in attenuating the harmful effects of high fructose and high glucose diets in different organs. This premise was based on the *in vitro* MG scavenging effect of arginine reported recently by our lab. My results with L-arginine were mixed with attenuation of increase

in MG in some organs and failure to attenuate in others. Measurement of plasma and organ/tissue levels of L-arginine would have enabled me to determine the effectiveness and adequacy of oral dose of L-arginine used in this study and its effective distribution in different organs after absorption. In this regard I should mention that our lab is developing a project to study the detailed pharmacokinetics of arginine. Lack of availability of samples of certain organs, such as the aorta and pancreas also prevented me from measuring the GSH levels in these tissues. Despite these limitations, I was able to generate some interesting data and gain very useful and extremely satisfying research experience from my study.

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